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 infiltration in lung adenocarcinoma (LUAD) are 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, and GEPIA database. The 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: The transcription levels of GRAP2 were significantly lower in several human cancer types, including LUAD, than in adjacent normal tissues. Immunohistochemistry staining confirmed that LUAD tumor tissues had lower GRAP2 protein expression levels than adjacent normal tissues. GRAP2 downregulation was associated with poorer overall survival, pathologic stage, T stage, N stage, and primary therapy outcome in LUAD. Mechanistically, we found a hub gene set that included a total of 91 genes co-expressed with GRAP2, which were closely related to the immune response in LUAD. The expression levels of GRAP2 were positively correlated with the infiltration levels of multiple immune cells and the cumulative survival time of a few immune cells. GRAP2 expression was found to be positively correlated with that of multiple immune markers, chemokines, chemokine receptors, and MHC molecules in LUAD.

Conclusions: GRAP2 can be used as a biomarker for assessing prognosis and immune infiltration levels in LUAD.

Keywords
GRAP2, immune infiltration, lung adenocarcinoma, prognostic biomarker
1 | INTRODUCTION

Lung cancer is one of the malignant tumors with the highest incidence and worst prognosis in the world.¹ Lung adenocarcinoma (LUAD) is a common subtype of lung cancer.²⁴ 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 against LUAD for diagnosis, prevention, and treatment.

The tumor microenvironment (TME) includes the extracellular matrix, stromal cells, and tumor-infiltrating immune cells (TIICs) that shape cancer development.⁶ Increasing evidence indicates that TIICs determine the success of immunotherapy and affect the prognosis of patients.⁷ Currently, which factors drive immune infiltration in LUAD is unclear. 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 in leukocytes.⁸ Studies have shown that GRAP2 is highly expressed in lymphoid organs and T lymphocytes. GRAP2 forms signaling complexes with different signaling molecules to mediate the activation and signal transduction of T cells.⁹

At present, research on GRAP2 mainly focuses on the immune system.¹⁰¹¹ 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, and the research indicates 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, an increasing number of platforms and open databases have enabled researchers to use multiple datasets for cancer bioinformatics analysis.¹³ To better explore the role of GRAP2 in LUAD, we assessed the relationship between GRAP2 expression and the prognosis of patients with LUAD using TCGA, TIMER, and the Kaplan–Meier plotter database. In addition, we investigated the correlation between GRAP2 expression and immune infiltration using 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

2.1 | Patient dataset

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) was downloaded from the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/gds/?term=).

2.2 | Survival analysis

The survival rate and hazard ratio (HR) analyses were performed using the Kaplan–Meier plotter database (http://kmplot.com/analysis) and Gene Expression Profiling Interactive Analysis (GEPIA) database (http://GEPIA.cancer-pku.cn).¹⁶

2.3 | Correlation heatmap and protein–protein interaction (PPI) network analysis

The GeneMANIA database (http://www.genemania.org)¹⁷ was used to generate a correlation heatmap for the genes coexpressed with GRAP2. The STRING database (https://string-db.org) was used to obtain PPI data based on protein interactions and signaling pathways, and Cytoscape 3.7.2 was used to construct the network.

2.4 | GEPIA database analysis

GEPIA (http://gepia.cancer-pku.cn/index.html)¹⁶ was used to screen genes that were positively or negatively correlated with GRAP2 by applying the “Similar Genes” module. The “Survival” module in GEPIA was used to explore genes associated with LUAD prognosis.

2.5 | Linked omics database analysis

The Linked Omics database (http://www.linkedomics.org)¹⁸ contains 32 tumor types from the TCGA database and multiomics data and clinical data of a total of 11,158 patients. In this study, the gene ontology biological process (GO_BP) and KEGG pathway analyses were performed using the Linked Omics database.

2.6 | Timer database analysis

In this study, the TIMER database (http://www.cistrome.shinyapps.io)¹⁹ was used to examine gene expression in normal tissues and tumor tissues from a variety of tumor types. Based on the gene expression profile of LUAD samples in TCGA data, the abundance of TIICs was also analyzed using the TIMER database.

2.7 | TISIDB database analysis

The TISIDB database (http://cis.hku.hk/TISIDB)²⁰ is a highly cited portal that allows researchers to interactively explore tumor and immune system interactions. In this study, the “Immunomodulator” module of the TISIDB database was utilized to explore the correlation between GRAP2 expression and MHC molecules. To investigate the relationship between GRAP2 and chemokine/chemokine receptor expression, we examined the chemokine/chemokine receptor expression level in TIICs using the “chemokine” module.
Figure 1: mRNA levels of GRAP2 in different human cancer types. (A) TIMER was used to detect the GRAP2 mRNA expression levels in different tumors. The GRAP2 mRNA expression level was detected in cancer tissues (n = 515) and adjacent normal tissues (B) unmatched tissues, n = 59; (C) matched tissues, n = 57) and in cancer tissues from patients with different clinical features in TCGA: (D) T stage (sample numbers: T1 = 175, T2 = 289, T3 = 49, T4 = 19), (E) N stage (sample numbers: N0 = 348, N1 = 95, N2 = 74, N3 = 2), (F) pathologic stage (sample numbers: Stage I = 294, Stage II = 123, Stage III = 26, Stage IV = 59), (G) primary therapy outcome (sample numbers: PD = 71, SD = 37, PR = 6, CR = 332). *p < 0.05, **p < 0.01, ***p < 0.001.
2.8 | Western blotting

LUAD cells were lysed using RIPA lysis buffer (MedchemExpress, China). The protein lysate was separated via SDS–PAGE (Invitrogen) and transferred to PVDF membranes (Millipore, USA) for analysis.21 The membranes were incubated with anti-GRAP2 (1:2000 dilution, Cambridge, MA, USA, ab224613) and anti-GAPDH (1:5000 dilution, Proteintech, China, 60,004-1-Ig) antibodies at 4°C overnight and then with HRP-labeled secondary antibody (1:2000 dilution, Beyotime, China, A0181) at room temperature for 2h. Western blotting results were analyzed with ImageJ software.

2.9 | Immunohistochemistry (IHC)

Seventy-six paraffin-embedded LUAD tissues and para-carcinoma tissues were used for IHC staining. Tissues were incubated with anti-GRAP2 antibody (1:2000 dilution, MA, USA, ab224613) overnight at 4 °C. After three washes with phosphate-buffered saline (PBS), the tissues were incubated with HRP-labeled goat anti-mouse secondary antibody (1:2000 dilution, Beyotime, China, A0181) for 0.5 h at room temperature. The stained IHC sections were counterstained with hematoxylin (Beyotime). Slides were scanned using a microscope (Fisher Scientific), and ImageJ software was utilized to analyze the intensity of staining.22

2.10 | Statistical analysis

The original data were calculated using GraphPad Prism software (version 8.0). Gene expression correlations were evaluated via Spearman’s correlation, and statistical significance was determined. Univariate and multivariate Cox analyses were used to screen for potential prognostic factors. A Mann–Whitney test and an independent t test were used to analyze differences between two groups of data. Differences among multiple groups were calculated using one-way ANOVA with a 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. The results showed that the GRAP2 transcriptional level was significantly lower in tumor tissues than in matched normal tissues, including in bladder urothelial carcinoma, breast invasive carcinoma, colon adenocarcinoma, LUAD, lung squamous cell carcinoma (LUSC), prostate adenocarcinoma, and rectum adenocarcinoma. However, higher transcriptional levels were 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 LUAD tumor tissues compared with adjacent normal tissues. 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 tissue (Figure 1C). Similar results were found in LUSC (Figure S1A,B).

### Table 1 Correlation between GRAP2 expression and the clinicopathological characteristics of LUAD cases from TCGA

| Characteristic         | Low expression of GRAP2 | High expression of GRAP2 | p       |
|------------------------|-------------------------|--------------------------|---------|
| n                      | 267                     | 268                      | <0.001  |
| T stage, n (%)         |                         |                          |         |
| 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   |
| <=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, median (IQR)      | 65 (58, 72)             | 66.5 (59, 72)            | 0.591   |
Table 1 summarizes the correlation between GRAP2 expression and clinical characteristics in LUAD. The results showed that low expression of GRAP2 was associated with late T stage, N stage, pathologic stage, and worse primary therapy outcome (Figure 1D–G and Table 1). The protein expression level of GRAP2 was further investigated by immunohistochemical staining, the clinicopathological features of the LUAD cases are presented in Supplementary Table S1, and we found that the GRAP2 protein level was obviously decreased in LUAD tissues compared with adjacent normal tissues (Figure 2A,B). Finally, we investigated GRAP2 expression in LUAD cell lines, and the data showed that GRAP2 mRNA levels were significantly downregulated in three LUAD cell lines (A549, H1975, and H1299) compared with levels in a normal 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 is correlated 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 GRAP2 expression exhibited good overall survival (OS) (HR = 0.61, p = 0.001) according to a Kaplan–Meier survival analysis (Figure 3A). However, the correlation between GRAP2 expression and OS in LUSC was not significant (HR = 0.95, p = 0.727) (Figure S1C). Therefore, we only analyzed the role of GRAP2 in LUAD in the follow-up study. Subgroup analysis showed that high GRAP2 expression was significantly associated with longer OS in LUAD under the following conditions: T2 stage (HR = 0.66, p = 0.035), N0 and 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–L). 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: HR = 0.602, 95% CI = 0.448–0.808, p < 0.001; multivariate Cox: HR = 0.602, 95% CI = 0.448–0.808, p < 0.001) (Figure 4A,B). Finally, we used an independent external GEO dataset, GSE37745, to verify our results, and the results were consistent with the above findings. LUAD patients with higher GRAP2 expression showed higher OS than patients with lower GRAP2 expression (Figure S2). 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 the immune response in LUAD

To examine the biological function of GRAP2 in LUAD, we used the GEPIA database to detect the coexpression pattern of GRAP2
FIGURE 3  Kaplan–Meier overall survival of patients with LUAD according to GRAP2 expression level. (A) Kaplan–Meier estimates of the effect of GRAP2 on OS in LUAD. (B–L) Subgroup analysis for T1, N0 and N1, M0, pathological staging stage III, PD and SD, residual tumor stage R0, smoker, male, female, age >60, age ≤60.

FIGURE 4  Forest plot of the univariate and multivariate Cox regression analyses in LUAD. (A) Univariate and (B) multivariate Cox regression analyses of the risk score and other clinical characteristics in the TCGA dataset.
in LUAD. In the heatmap in Figure 5A, the first 25 genes (top 25) were positively correlated with GRAP2 expression, and the last 25 genes (bottom 25) were negatively correlated with GRAP2 expression. We used the “Link Interpreter” module of the Linked Omics website to evaluate functional enrichment among the genes coexpressed with GRAP2 (top 600) using GO and KEGG annotations. GO annotations showed that these genes were enriched in immune response processes, such as Th17 cell differentiation, T-cell activation, initial immune deficiency, and cytokine receptor activation (Figure 5B). KEGG annotations showed that GRAP2 may be associated with multiple signaling pathways in cancer, including the chemokine signaling pathway, T-cell receptor signaling pathway and PD-L1 expression, and PD-1 checkpoint pathway (Table S2). These data suggest that GRAP2 is closely related to the regulation of immune cell function.

We also performed GO and KEGG annotation analyses in LUSC. The data showed that enriched pathways in the genes coexpressed with GRAP2 (top 600) were closely associated with the immune response in LUSC, but there were few overlapping enrichment items between LUAD and LUSC (Figure S3).

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

We further used protein–protein interactions (PPIs) 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 the proteins in the network had a strong positive correlation with each other (Figure 6B). Therefore, these established genes coexpressed with GRAP2 are particularly related to the immune response, which may be the molecular mechanism by which GRAP2 affects the prognosis of patients with LUAD. Moreover, these coexpressed genes can potentially be used in a multigene biomarker panel to predict survival in LUAD.

3.4 | The expression level of GRAP2 is correlated with 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 LUAD cases into two cohorts according to the expression level of GRAP2 and estimated the immune score using the ESTIMATE database. The immune score was significantly higher in the cohort with high GRAP2 expression than in the cohort with low GRAP2 expression (Figure 7A).

Studies have shown that the survival time of many cancer patients is determined by the number and activity status of TIIICs. 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 levels in the cohort with high GRAP2 expression (Figure 7B). Furthermore, the GRAP2 expression level was negatively correlated with tumor purity; however, it was positively correlated with the 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 ($r = 0.252, p < 0.001$), 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 the cohort with high GRAP2 expression had a higher cumulative survival time of B cells ($p = 0$) and dendritic cells ($p = 0.048$) but not CD8+ T cells, CD4+ T cells, neutrophils, or 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 further explore the relationship between GRAP2 and the immune response, the TIMER database was used to investigate correlations between GRAP2 expression and diverse immune markers in LUAD. These immune markers can be used to characterize immune cells, including T cells (general), CD8+ T cells, Th1 cells, Th2 cells, follicular helper T cells, Th17 cells, Tregs, effector T cells, effector memory T cells, resident memory T cells, general memory T cells, exhausted T cells, B cells, monocytes, neutrophils, natural killer cells, and dendritic cells. In clinical cancer biopsies, tumor purity is an important parameter reflecting the level of immune infiltration.

Our data showed that GRAP2 expression was positively correlated with most immune markers in multiple types of immune cells, and 58 of 59 immune markers presented a positive correlation with GRAP2 expression in LUAD (Table 2). These data further support the notion that GRAP2 expression is significantly correlated with immune infiltration.
3.6 | GRAP2 is positively correlated with chemokine/chemokine receptors and MHC molecules

Chemokines and chemokine receptors play important roles in the infiltration of immune cells into tumors. To investigate the potential mechanism by which GRAP2 regulates immune infiltration in LUAD, the TISIDB database was used to examine correlations between the expression level of GRAP2 and those of chemokines/chemokine receptors. A heatmap showed that GRAP2 expression was positively correlated with that of various chemokines and chemokine receptors in multiple tumor types (Figure 8A,C). We also comprehensively explored the correlation between GRAP2 expression and chemokine/chemokine receptors using scatter plots. The 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). The 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) promotes tumor immune suppression, metastatic progression, and a poor prognosis in multiple tumor types. Therefore, the TISIDB database was used to explore the correlation between GRAP2 expression and MHC molecules. Heatmap results showed that the MHC molecules in numerous tumors were significantly positively correlated with the expression of GRAP2 (Figure 9A). Scatter plots also showed that many MHC molecules were positively correlated

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**FIGURE 7** Correlation analysis of GRAP2 expression and immune infiltration in LUAD. (A) The correlation between immune score and GRAP2 expression according to the ESTIMATE database (sample numbers: GRAP2 low = 257, GRAP2 high = 258). (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. *p < 0.05, **p < 0.01, ***p < 0.001.
TABLE 2 Correlation analysis between GRAP2 expression and immune markers using TIMER

| Description         | Gene markers | None       | Purity     |
|---------------------|--------------|------------|------------|
|                     |              | Cor  | p     | Cor  | p     |
| T cell (general)    | CD3D         | 0.728 | ***   | 0.654 | ***   |
|                     | CD3E         | 0.81  | ***   | 0.768 | ***   |
|                     | CD2          | 0.817 | ***   | 0.773 | ***   |
| CD8+ T cell         | CD8A         | 0.727 | ***   | 0.674 | ***   |
|                     | CD8B         | 0.646 | ***   | 0.593 | ***   |
| 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 | ***   |
| Effector T cell     | CX3CR1       | 0.381 | ***   | 0.324 | ***   |
| Effector memory T cell | DUSP4    | -0.023 |       | -0.032 |       |
|                     | G2MK         | 0.737 | ***   | 0.673 | ***   |
|                     | G2MA         | 0.681 | ***   | 0.613 | ***   |
| Resident memory T cell | CD69     | 0.683 | ***   | 0.617 | ***   |
|                     | CXCR6        | 0.78  | ***   | 0.729 | ***   |
| General memory T cell | CCR7      | 0.669 | ***   | 0.578 | ***   |
|                     | SELL         | 0.621 | ***   | 0.527 | ***   |
| Effected T cell     | HAVCR2       | 0.559 | ***   | 0.456 | ***   |
|                     | LAG3         | 0.576 | ***   | 0.509 | ***   |
|                     | CXCL13       | 0.092 | *     | -0.016 |       |
| 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 | ***   |
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–Q). These data suggest that GRAP2 participates widely in modulating multiple immune-related molecules in LUAD to affect immune infiltration and the immune response in the TME.

4 | DISCUSSION

GRAP2 is an adaptor protein in the GRB2 family. Studies have shown that GRAP2 plays an important role in the development and function of T cells. GRAP2 needs to form a complex with other proteins to trigger the activation of downstream signaling molecules, and 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. At present, the role of GRAP2 in tumors is unclear. In this study, TCGA, TIMER, and TISIDB public databases were used to perform bioinformatics analyses, and the GRAP2 gene showed significantly lower expression in LUAD than in adjacent normal tissue. In the prognostic analysis, we found that downregulation of GRAP2 was associated with poor OS. At the same time, low GRAP2 expression was associated with poor clinicopathological characteristics. These findings indicate that GRAP2 can be used as a tumor suppressor gene and a prognostic biomarker. To explore the underlying molecular mechanism by which GRAP2 affects the prognosis of patients with LUAD, we identified 91 genes from among the genes coexpressed with GRAP2 as 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 downregulated in LUSC but has no association with OS. Although the expression of GRAP2 is downregulated in both LUAD and LUSC, the functions of GRAP2 may be different in each cancer type. Our GO and KEGG analyses showed a few overlapping enrichment items between LUAD and LUSC. Nevertheless, in both LUAD and LUSC, GRAP2 was found to be closely associated with signaling pathways related to the immune response.

Cancer cells have evolved multiple mechanisms to evade immune attack, including dysfunction of tumor antigen presentation, and recruitment of immunosuppressive cells. An increasing number of studies have demonstrated that different populations of immune cells show distinct effects on tumor progression and on-therapy. Reports have shown that CD8+ T cells, NK cells, and perhaps NKT cells may mediate antitumor immunity, whereas Treg cells, myeloid-derived suppressor cells, Th22 cells, and perhaps B cells may promote tumorigenesis. 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 the recruitment of immune cells into the TME, including both antitumor subsets and protumor subsets. Therefore, the precise role of GRAP2 in the TME needs further exploration.

Chemokines are secreted proteins with a low molecular weight that mainly mediate immune cell trafficking and lymphoid tissue development. Immune cells are recruited into the TME by interactions between chemokines and paired chemokine receptors. In this study, we detected the correlation between GRAP2 expression

| TABLE 2 (Continued) |
|----------------------|----------------|----------------|
| Description          | None           | Purity         |
| Gene markers         | Cor    | p     | Cor    | p     |
| 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  | ***  |

*p < 0.05, **p < 0.01, ***p < 0.001.
and chemokines and chemokine receptors in LUAD. GRAP2 expression was found to be significantly correlated with various chemokines and chemokine receptors. These findings may explain how GRAP2 regulates immune infiltration in LUAD.

MHC molecules participate in antigen recognition in the immune response.\textsuperscript{36} Simple antigens cannot activate immune cells. Antigens are degraded by cytosolic and nuclear proteasomes and bound to antigen-binding sites in MHC molecules on the surface of antigen-presenting cells; then, the antigens can be recognized by T and B cells.\textsuperscript{37} The expression of MHC on the surface of tumor cells represents the characteristics of tumor cells.\textsuperscript{38} The poorer the differentiation of tumor cells, the weaker the expression of MHC molecules, which would result in

\textbf{FIGURE 8} Correlation analysis between GRAP2 expression and chemokines and/or chemokine receptors. (A and C) Heatmap analysis of the correlation between GRAP2 expression and chemokines/chemokine receptors in tumors. (B and 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 shown by heatmap analysis. (B-Q) Correlation between GRAP2 expression and MHC molecules in LUAD shown by scatter diagram analysis.
immune escape of tumor cells. Here, our data indicate that GRAP2 expression is positively correlated with numerous MHC molecules. These data strongly suggest that GRAP2 plays an important role in the presentation of tumor antigens in LUAD.

However, despite our systematic analysis of GRAP2, there are still some limitations in this study. First, in vivo/in vitro experiments are needed to demonstrate the effect of GRAP2 on tumor progression to improve the reliability of our results. Second, although we concluded that GRAP2 expression is closely related to immune infiltration in LUAD and patient prognosis, we do not have direct evidence that GRAP2 influences prognosis by regulating immune infiltration. These problems deserve further experimental verification in the future. Third, this study is mainly based on public databases, and thus, the quality and uniformity of the data from different databases can affect the interpretation of the research results. However, the analysis of data from multiple databases produced similar results, which supports the conclusions of our study.

5 CONCLUSION

We conclude that GRAP2 is a tumor suppressor gene and can potentially be used as a prognostic marker in LUAD. Decreased GRAP2 expression is correlated with poor clinical characteristics and low immune infiltration. These findings could benefit further evaluation of the effect of GRAP2 in LUAD and reveal potential targets for LUAD subclasses for individual prognosis determination and treatment. Perhaps, in further research, we can assess the degree of malignancy of LUAD by detecting the expression level of GRAP2 in surgical specimens of LUAD and even better evaluate the status of the TME.

AUTHOR CONTRIBUTIONS

ZL and YY contributed to study concept and design and study supervision. SS and XD contributed to acquisition of data and drafting of the article. SJ and CT contributed to analysis and interpretation of data. JH and NL contributed to statistical analysis. XD, YY, JC, and ZL obtained funding. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

DATA AVAILABILITY STATEMENT

All data are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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