# LIMK1: A Promising Prognostic and Immune Infiltration Indicator in Colorectal Cancer

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**Research Article**

**Keywords:** LIMK1, Colorectal cancer, Immune infiltration, Prognosis, Biomarker

**Posted Date:** February 21st, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1360875/v1

## Abstract

# Background

Studies have shown that LIMK1 is upregulated in a variety of tumors and may be a potential target for detection. In this study, we analyzed the expression difference of LIMK1 and its relationship with tumor clinicopathological characteristics and tumor microenvironment in colorectal cancer combining public databases and experimental validation.

# Methods

The transcriptomic data of LIMK1 with colorectal cancer were downloaded from The Cancer Genome Atlas (TCGA) database and GEO databases to analyze the expression of LIMK1 mRNA and its correlation with patient prognosis. The protein expression of LIMK1 was obtained from Human Protein Atlas. We used receiver operating characteristic (ROC) curves and Kaplan-Meier curves to evaluate the expression characteristics and prognostic differences of LIMK1 in colorectal cancer. STRING was used to analyze the co-expression of LIMK1. The tumor immune estimation resource (TIMER) was used to determine the correlation between LIMK1 expression and immune infiltrates. We verified LIMK1 expression at the level of clinical samples collected from the Tianjin Medical University General Hospital and cell lines using qRT-PCR.

# Results

LIMK1 is differentially expressed in different cancer types. The mRNA and protein expression of LIMK1 were upregulated in tumor tissues compared with adjacent tissues in colorectal cancer. The expression level of LIMK1 was positively correlated with the clinicopathological features of colorectal cancer, disease-specific survival (DSS) events ($P = 0.002$), and lymphatic invasion ($P = 0.004$). The AUC value of LIMK1 in colorectal cancer was 0.937 (95% confidence interval [CI]: 0.918–0.957) through ROC analysis. Under the best cut-off value (4.009), the sensitivity and specificity were 98% and 81.9%, respectively. LIMK1 expression is mainly related to $CD4^+$ T cells, macrophages, and dendritic cells in the immune microenvironment of CRC.

# Conclusions

The high expression of LIMK1 in colorectal cancer is closely related to the clinical features and prognosis of patients. LIMK1 is a promising prognostic indicator that may regulate tumor progression by affecting tumor immune microenvironment in colorectal cancer.

## Introduction

Colorectal cancer (CRC) is one of the most common and lethal malignancies worldwide. More than 1.2 million new CRC cases were reported globally in 2020 according to cancer prevalence statistics[1]. Due to the impact of COVID-19 in 2020, poor medical conditions will certainly lead to a higher fatality rate in the future. With the popularization of people's health awareness, the detection rate of CRC is increasing. However, the metastatic rate of colorectal cancer remains high, especially liver metastasis of colorectal cancer, which leads to a low 5-year survival rate of colorectal cancer[2]. Currently, CRC screening trials mainly rely on colonoscopy and some blood tests such as CEA (carcinoembryonic antigen), carbohydrate antigen 19–9 (CA19–9), and fecal occult blood test (FOBT)[3]. Although many new molecular targets continue to be discovered as diagnostic and predictive biomarkers for CRC, such as microRNAs and circular RNAs[4, 5], there are still many challenges that hinder the clinical practice for real applications. Thus, there is an urgent need to identify new biomarkers that can accurately predict CRC, especially metastatic CRC, to improve the prognosis and curative effect of adenocarcinoma.

LIM domain kinase 1 (LIMK1) is a kinase of the LIMK family and consists of two related proteins, LIMK1 and LIMK2[6, 7]. LIMK1 is mainly expressed in the cytoplasm and in small amounts in the nucleus. LIMK1 promotes actin polymerization and phosphorylates its downstream target cofilin, which further influences cell growth and functions, including cell proliferation, angiogenesis, and cell cycle progression[8, 9]. In the initiation and progression of tumor, lots of studies have verified that the abnormal expression of LIMK1 was closely related to the change of biological behavior of several human tumors, especially prostate cancer, breast cancer and gastric cancer, and so forth[10–13]. A study by Zhang et al. indicated that lung carcinoma cell proliferation and tumor metastasis were suppressed by inhibiting LIMK1 activity in vivo and in vitro[10]. In a subsequent bioinformatics analysis, it was reported that the expression of LIMK1 was significantly correlated with tumor-infiltrating immune cells (IC) and poor prognosis of lung cancer[14]. In CRC, a few recent studies have demonstrated that upregulation of LIMK1 through direct or indirect pathways can promote colorectal cancer cell proliferation, invasion, and migration in vitro[15, 16]. Therefore, LIMK1 may be a suitable biomarker for the diagnosis and treatment of CRC.

Although the fact that regulation of LIMK1 expression can change malignant biological behaviors such as proliferation and migration of CRC[15], the relationship between LIMK1 and the immune microenvironment has not been reported in CRC. We speculated that LIMK1 might influenced immune cells or other immune markers in CRC. To prove this conjecture, we first explored LIMK1 the differential and prognostic value of CRC based on data from The Cancer Genome Atlas (TCGA) database and some online websites. We found that LIMK1 is indeed upregulated in CRC. We verified this conclusion using the Gene Expression Omnibus database (GEO) and clinical samples from our center. Moreover, the expression of LIMK1 was associated with multiple clinicopathological features of CRC patients. We further evaluated the relationship between LIMK1 and immune-related indicators in CRC. In summary, we analyzed the relationship between high expression of LIMK1 and CRC from different perspectives.

# Results

# mRNA expression differences and prognosis analysis of LIMK1 in pan-cancer

As mentioned in previous studies, LIMK1 is differentially expressed in a variety of cancer types. To determine the intracellular localization of LIMK1, we studied the distribution of LIMK1 in three tumor cell lines according to the HPA database. LIMK1 was expressed in varying degrees in the nucleus and cytoplasm (Fig. 1A-1B). LIMK1 also had different levels of RNA expression in cell lines of different tissues (Fig. 1C). We analyzed the expression of LIMK1 in 33 cancers and the corresponding paracancer types. As shown in Fig. 1D, the expression of LIMK1 was significantly upregulated in 12 types of tumors, including colon cancer, rectal cancer, lung cancer, and stomach cancer. However, LIMK1 was downregulated in LGG ($P < 0.01$). Therefore, we confirmed that LIMK1 is differentially expressed in different cancer types. Subsequently, to verify whether the differential expression of LIMK1 was related to the patient's survival prognosis, we analyzed the prognostic indicators of patients with differential expression of LIMK1. As shown in Fig. 2, the results showed that the overall survival of COAD (HR = 1.55, *P* = 2.60e-02), KIRP (HR = 2.57, *P* = 2.00e-03), LGG (HR = 2.39, *P* = 1.00e-03), LUAD (HR = 1.38, *P* = 2.90e-02), and READ (HR = 1.22, *P* = 2.20e-02) were significantly different.

# The expression of LIMK1 mRNA and protein in CRC

To further examine the differential expression of LIMK1, we analyzed the expression of LIMK1 mRNA and protein in CRC tissues using data from the TCGA database and TPA. Paired and unpaired samples from the TCGA database were analyzed (Additional file 1 and 2). As shown in Fig. 3A-3B, analysis of paired data showed that the expression level of LIMK1 mRNA in CRC tissues (50) was significantly higher than that in normal tissues (50). The average level of the normal group was 3.43 ± 0.353, and that of the tumor group was 4.662 ± 0.535 (paired t-test, *P* = 1.12e-03). Analysis of unpaired data also showed that the expression level of LIMK1 mRNA in CRC tissues (647) was significantly higher than that in normal tissues (51). The average level of the normal group was 3.434 ± 0.351, and that of the tumor group was 4.501 ± 0.620 (Mann-Whitney U-test, *P* = 1.08e-03). As shown in Fig. 3C-3D, LIMK1 protein expression was also upregulated in CRC tissues based on the immunohistochemical staining results from TPA.

# Validation of LIMK1 mRNA differentially expressed level in CRC

In order to further prove the difference in LIMK1 expression in the TCGA database, we analyzed the expression level of LIMK1 in four GEO datasets (GSE 10715, GSE 18105, GSE 22598, and GSE 32323) and detected LIMK1 expression levels in CRC samples and peritumoral non-cancerous tissues obtained from Tianjin medical university generral hospital by qRT-PCR. As shown in Fig. 4A-4D, LIMK1 was significantly upregulated in CRC tissues compared to that in normal tissues. As shown in Fig. 4E-4F, in cell-level verification, the expression level of LIMK1 was significantly increased in several CRC cell lines compared to the normal colonic epithelium cell line (Additional file 3). And the LIMK1 expression levels in CRC and para-cancerous tissues were detected by qRT-PCR. The relative mRNA expression of LIMK1 was

upregulated in CRC tissues (Additional file 4) compared to that in normal tissues (*P* = 3.20e-04). The above results confirmed that LIMK1 had obvious differences in expression in CRC.

# Correaltion between the expression of LIMK1 mRNA and clinicopathological features of CRC patients

To verify the relationship between LIMK1 expression and clinical indicators in patients, we explored the relationship between LIMK1 mRNA levels and the clinicopathological features of CRC patients. As shown in the Table. 1 and Fig. 5, the expression level of LIMK1 was positively correlated with some clinical pathological features of CRC, such as disease-specific survival (DSS) events (*P* = 2.00e-03) and lymphatic invasion (*P* = 4.00e-03). Although it did not make sense to analyze pathologic stage I-IV alone, patients who belonged to stage III-IV were highly expressed in LIMK1 than in stage I-II (*P* = 4.20e-02). There were no statistical differences in the rest of the clinicopathological characteristics. Overall, the above results suggest that LIMK1 could indicate the prognosis of CRC patients in some respects.

**Table 1**

**Correlation between LIMK1 expression and pathological parameters of CRC patients.**

| Characteristic | Low expression of LIMK1 | High expression of LIMK1 | *P*-value |
|---|---|---|---|
| n | 322 | 322 | |
| Gender | | | 1.000 |
| Female | 148 (23%) | 153 (23.8%) | |
| Male | 174 (27%) | 169 (26.2%) | |
| Age | | | 1.000 |
| <=65 | 138 (21.4%) | 138 (21.4%) | |
| > 65 | 184 (28.6%) | 184 (28.6%) | |
| T stage | | | 0.341 |
| T1 | 9 (1.4%) | 11 (1.7%) | |
| T2 | 63 (9.8%) | 48 (7.5%) | |
| T3 | 208 (32.4%) | 228 (35.6%) | |
| T4 | 39 (6.1%) | 35 (5.5%) | |
| N stage | | | 0.076 |
| N0 | 195 (30.5%) | 173 (27%) | |
| N1 | 74 (11.6%) | 79 (12.3%) | |
| N2 | 49 (7.7%) | 70 (10.9%) | |
| M stage | | | 0.408 |
| M0 | 234 (41.5%) | 241 (42.7%) | |
| M1 | 39 (6.9%) | 50 (8.9%) | |
| Lymphatic invasion | | | 0.004 |
| No | 196 (33.7%) | 154 (26.5%) | |
| Yes | 101 (17.4%) | 131 (22.5%) | |
| Pathologic stage | | | 0.306 |
| Stage I | 59 (9.5%) | 52 (8.3%) | |
| Stage II | 127 (20.4%) | 111 (17.8%) | |
| Stage III | 84 (13.5%) | 100 (16.1%) | |
| Stage IV | 41 (6.6%) | 49 (7.9%) | |
| BMI | | | 0.111 |

| Characteristic | Low expression of LIMK1 | High expression of LIMK1 | *P*-value |
|---|---|---|---|
| < 25 | 49 (14.9%) | 58 (17.6%) | |
| >=25 | 124 (37.7%) | 98 (29.8%) | |
| CEA level | | | 0.069 |
| <=5 | 139 (33.5%) | 122 (29.4%) | |
| > 5 | 67 (16.1%) | 87 (21%) | |
| DSS event | | | 0.002 |
| Alive | 289 (46.5%) | 255 (41%) | |
| Dead | 26 (4.2%) | 52 (8.4%) | |
| BMI, median (IQR) | 27.46 (24.77, 32.86) | 26.51 (23.31, 31.08) | 0.028 |

# The relationship between LIMK1 and prognosis in patients with CRC

To investigate the relationship between LIMK1 expression and survival in patients with CRC, Kaplan-Meier curves were generated. As shown in Fig. 6A-6C, although the progress free interval (PFI) was not significant ($P$ = 0.211), the overall survival (OS) of CRC patients with lower LIMK1 expression levels was longer than that of patients with higher LIMK1 expression levels ($P$ = 3.90e-02). The same was true for DSS ($P$ = 4.00e-03). Subsequently, we performed ROC curve analysis to determine whether LIMK can distinguish between CRC samples and normal samples. As shown in Fig. 6D, the AUC of LIMK1 was 0.937 (95% CI: 0.918–0.957), according to the ROC curve. When each predictive variable was at its optimum cut-off value (cut-off = 4.009), the sensitivity and specificity were 98% and 81.9%, respectively. These results suggest that LIMK1 may be a promising biomarker for CRC diagnosis.

# The PPI networks and functional annotations of LIMK1

Next, we tried to determine the PPI networks and functional annotations of LIMK1 using GO, KEGG, and STRING databases. We analyzed the top 10 co-expression genes of LIMK1 and constructed a network according to the distance of the relationship shown in Fig. 7A. As shown in Fig. 7B, the cellular component (CC) of LIMK1 and its co-expression genes mainly focused on lamellipodium, ruffle, and cell leading edge. Molecular function (MF) was associated with rho GTPase binding, ras GTPase binding, and protein serine/threonine kinase activity. The biological process (BP) was correlated with regulation of the actin filament-based process, regulation of actin cytoskeleton organization, and actin filament organization. KEGG analyses indicated that these interrelated genes mainly concentrated on axon guidance, regulation of actin cytoskeleton, and pathogenic E. coli infection pathways. The specific correlation of LIMK1 and its top 10 co-expression genes is shown in Fig. 7C-7K. Among them, CFL1, RHOB, and RHOC had the most significant correlation with LIMK1.

# The relationship of LIMK1 expression and immune Cell infiltration in CRC

To analyze the relationship between LIMK1 expression and immune cell infiltration in CRC, we calculated the correlation between LIMK1 and six immune cells using the TIMER database. Because the TIMER database did not have CRC data, we calculated the correlation between LIMK1 and the six immune cells in COAD and READ, respectively. As we can see that the correlation between LIMK1 expression and immune cell infiltration in COAD was almost equivalent to READ. LIMK1 expression was mainly related to $CD4^+$ T cells, macrophages, and dendritic cells (Fig. 8). Therefore, LIMK1 may play a role in the tumor immune response in CRC.

# Correlation analysis between LIMK1 expression and immune cell markers

To explore the correlation between LIMK1 and multiple immune infiltrating cells, we further analyzed the changes in different immune cell subgroups. We analyzed the relationship between LIMK1 and the immune markers of diverse cells in the GEPIA database. We observed that LIMK1 was indeed related to these immune cells Table. 2. In addition, LIMK1 seemed to be more related to M2 macrophages according to the correlation results. We also analyzed diverse T cell subsets in COAD and READ, such as Th1, Th2, Tfh, Th17, Tregs, and T cell exhaustion. In the analysis of T cell subpopulations, the correlation of Tregs and T cell exhaustion marker genes seemed to be higher than others.

**Table 2**

**Correlation analysis of LIMK1 and immune cell gene markers in GEPIA.**

| Description | Gene marker | COAD | | | READ | | |
|---|---|---|---|---|---|---|---|
| | | r | *P* | | r | *P* | |
| Th1 | T-bet (TBX21) | 0.31 | 1.90E-07 | *** | 0.45 | 5.70E-06 | *** |
| | STAT4 | 0.3 | 2.70E-07 | *** | 0.37 | 3.20E-04 | ** |
| | STAT1 | 0.35 | 2.10E-09 | *** | 0.6 | 2.50E-10 | *** |
| | IFN-γ (IFNG) | 0.21 | 4.40E-04 | ** | 0.45 | 5.50E-06 | *** |
| | TNF-α (TNF) | 0.25 | 2.50E-05 | *** | 0.44 | 9.00E-06 | *** |
| Th2 | STAT6 | 0.2 | 7.00E-04 | ** | 0.26 | 1.20E-02 | |
| | STAT5A | 0.4 | 9.50E-12 | *** | 0.28 | 6.50E-03 | ** |
| | IL13 | 0.22 | 3.10E-04 | ** | 0.093 | 3.80E-01 | |
| Tfh | BCL6 | 0.42 | 6.60E-13 | *** | 0.44 | 1.00E-05 | *** |
| | IL21 | 0.18 | 3.00E-03 | * | 0.15 | 1.60E-01 | |
| Th17 | STAT3 | 0.32 | 4.70E-08 | *** | 0.3 | 3.30E-03 | ** |
| | IL17A | -0.09 | 1.30E-01 | | 0.004 | 9.70E-01 | |
| Treg | FOXP3 | 0.44 | 1.50E-14 | *** | 0.46 | 4.10E-06 | *** |
| | CCR8 | 0.43 | 7.20E-14 | *** | 0.41 | 5.80E-05 | *** |
| | STAT5B | 0.36 | 4.60E-10 | *** | 0.36 | 9.30E-04 | ** |
| | TGFβ | 0.42 | 3.80E-13 | *** | 0.52 | 1.40E-07 | *** |
| T cell exhaustion | PD-1 (PDCD1) | 0.28 | 3.10E-06 | *** | 0.41 | 5.90E-05 | *** |
| | CTLA4 | 0.34 | 8.30E-09 | *** | 0.18 | 9.00E-02 | |
| | LAG3 | 0.13 | 3.50E-02 | | 0.35 | 5.50E-04 | ** |
| | TIM-3 | 0.44 | 1.90E-14 | *** | 0.55 | 1.90E-08 | *** |
| | GZMB | 0.02 | 6.90E-01 | | 0.14 | 1.70E-01 | |
| M1 | INOS (NOS2) | 0.13 | 3.50E-02 | | 0.35 | 5.50E-04 | ** |
| | IRF5 | 0.35 | 2.10E-09 | *** | 0.42 | 2.90E-05 | *** |
| | COX2 | 0.23 | 1.30E-04 | ** | 0.27 | 9.10E-03 | ** |
| M2 | CD163 | 0.34 | 5.80E-09 | *** | 0.48 | 1.10E-06 | *** |

| Description | Gene marker | COAD | | | READ | | |
|---|---|---|---|---|---|---|---|
| | | r | *P* | | r | *P* | |
| | VSIG4 | 0.41 | 1.20E-12 | *** | 0.37 | 2.70E-04 | ** |
| | MS4A4A | 0.4 | 5.00E-12 | *** | 0.46 | 4.10E-06 | *** |
| Dendritic cell | HLA-DPB1 | 0.36 | 4.50E-10 | *** | 0.35 | 6.10E-04 | ** |
| | HLA-DQB1 | 0.2 | 7.60E-04 | ** | 0.067 | 5.30E-01 | |
| | HLA-DRA | 0.31 | 1.80E-07 | *** | 0.38 | 2.20E-04 | ** |
| | HLA-DPA1 | 0.33 | 2.90E-08 | *** | 0.31 | 2.30E-03 | * |
| | BDCA-1 | 0.33 | 1.30E-08 | *** | 0.058 | 5.80E-01 | |
| | BDCA-4 | 0.46 | 8.90E-16 | *** | 0.48 | 1.10E-06 | *** |
| | CD11c | 0.42 | 3.60E-13 | *** | 0.38 | 1.90E-04 | ** |

# LIMK1 expression is associated with immune checkpoint genes in CRC

As shown in Fig. 9, we explored the correlation between LIMK1 expression and approximately 50 immune checkpoint genes in CRC. Our results showed that LIMK1 expression was positively correlated with the expression levels of various immune checkpoint genes in CRC (Additional file 5). Among them, the most relevant genes were CD276 (r = 0.558), TNFRSF4 (r = 0.440), and VSIR (r = 0.436), suggesting that LIMK1 may play a significant role in modulating tumor immunity by regulating these immune checkpoint genes.

# Discussion

CRC has a high morbidity and mortality rate, which urgently requires a robust molecule to achieve early diagnosis and treatment. The LIMK protein family includes LIMK1 and LIMK2. It was reported that LIMK1 was highly expressed in a variety of tumors and is related to patient prognosis. Although studies have shown that LIMK1 is up-regulated in CRC and causes poor prognosis[17]. And the related mechanism of LIMK1 regulating CRC progression has been studied[18]. But, the relationship between LIMK1 and tumor immune microenvironment in CRC has not been explored. In this article, we not only discuss the relationship between LIMK1 and CRC from several aspects such as expression level in tumor samples, clinicopathological features, but also the correlation with immune cell infiltration and the expression of immune checkpoint genes. First, we confirmed that the expression of LIMK1 mRNA and protein was higher in CRC tissues than in normal tissues. Subsequently, we found that higher LIMK1 expression was correlated with poor prognosis of CRC, regardless of OS or DSS. Moreover, LIMK1 can influence immune cell infiltration and immune checkpoint expression in CRC. In summary, LIMK1 may be a valuable and

promising biomarker for the diagnosis of CRC. And our findings laid the foundation of that LIMK1 promotes CRC progression from the mechnisim of regulating tumor immune microenvironment.

LIMK1, a serine protein kinase, is a member of the LIMK kinase family and plays a crucial role in the reorganization of actin and microtubule depolymerization[7]. Currently, research on LIMK1 has also focused on oncology because of its vital role in promoting tumor cell proliferation, invasion, and metastasis[19]. It has been reported that LIMK1 expression was upregulated in several kinds of human cancers especially in highly malignant neoplasm, such as lung adenocarcinoma, breast cancer and prostate cancer[11, 19, 20]. Furthermore, upregulated LIMK1 is often associated with poor patient prognosis. At present, lots of studies provied that LIMK1 was a significent biomarker which portends a poor prognosis in numerous cancer types. A study by Huang et al. [11]indicated that upregulation of LIMK1 was highly associated with lymph node metastasis and shortened biochemical-free survival in prostate cancer. In ovarian carcinoma, it was reported that high levels of LIMK1 indicate poor tumor differentiation and disease severity[21]. In gastric cancer, You et al. confirmed that with the upregulation of LIMK1, the size of the primary tumor was larger and the number of lymph node metastases was greater [22]. Moreover, it has been confirmed that reducing the expression of LIMK1 can delay tumor growth and peritoneal metastasis in vivo. In CRC research, upregulation of LIMK1 enhanced the invasiveness of CRC cells in vitro and in vivo[18]. In the present study, we demonstrated that LIMK1 mRNA is highly expressed in a variety of tumor tissues, including CRC. LIMK1 was highly expressed in tumor tissues with CRC, whether in paired or unpaired samples. Subsequently, we further indicated that the protein expression of LIMK1 was upregulated in CRC tumor tissues compared to that in adjacent tissues. Moreover, we confirmed that the upregulation of LIMK1 was associated with poor survival. Regarding clinicopathological characteristics, a significantly positive correlation was found between LIMK1 and lymphatic invasion, high TNM stage. Thus, LIMK1 might be more advantageous in the detection of metastatic CRC compared to previous screening methods such as CEA, FOBT, CA199, etc. To prove the accuracy and sensitivity of LIMK1 in the diagnosis of CRC, we performed ROC curve analysis. Our results indicated that the AUC value of LIMK1 was obviously high in the detection of CRC, with 98% sensitivity and 81.9% specificity. Although further studies are needed, LIMK1 might serve as a promising marker for identifying CRC with a poor prognosis.

LIMK1 plays an important role in several signaling pathways, especially those related to tumors. In our study, we analyzed the top 10 co-expression genes that were most related to the expression of LIMK1, of which CFL1, RHOB, and RHOC had the highest correlation. In addition, we found that LIMK1 is involved in a variety of biological processes in the following functional annotations. Zeng et al. [23] found that knockdown of Rho GDP dissociation inhibitor 2 (RhoGDI2) can downregulate the malignant biological behavior of gastric cancer cells via the Rac1/Pak1/LIMK1 pathway. In pancreatic cancer, other researchers have indicated that DEP domain-containing protein 1 B (DEPDC1B) can also stimulate cell migration and invasion through this pathway[24]. In functional annotations of LIMK1, we found that LIMK1 was mainly focused on lamellipodium, ruffle, and cell leading edge in CC. The accumulation of LIMK1 in these cellular components was likely to indicate that it was related to the migration and metastasis of tumor cells. Vainer et al. [25]found VICKZ accumulated at the leading edge of SW480 CRC

cells which facilitated the formation of surface morphologies required for cell migration. Rho GTPase-activating protein 5 (ARHGAP5) promoted EMT to accelerate tumor metastasis through regulating RhoA activity in CRC cell[26]which corroborated our results of LIMK1 enrichment in MF. And axon guidance[27], regulation of actin cytoskeleton[28], and pathogenic E. coli infection[29]pathways were inovoled in the process of the occurrence or metastasis in CRC. Therefore, combined with our results, we speculate that the upregulated LIMK1 may directly or indirectly affect the biological functions of tumors by regulating these proteins and pathways.

Another novelty of this study was that LIMK1 expression was associated with immune cell infiltration in CRC. Several recent studies have shown that LIMK1 may be involved in the regulation of the immune microenvironment. In T cell immunity, HIV triggers actin polymerization through the LIMK1-cofilin signaling pathway[30]. In NK cells, Duvall et al. [31] identified LIMK1 as a vital medium to regulate cytoskeletal rearrangement. However, the role of LIMK1 in the immune tumor microenvironment has not been extensively discussed. Only some researchers have found that the expression of LIMK1 is enriched in immune (CMS1) subtypes of CRC[17]. In our study, we found that LIMK1 was associated with multiple tumor-infiltrating immune cells in CRC. Among them, LIMK1 was most closely related to $CD4^+$ T cells, macrophages, and dendritic cells. In further subgroup analysis, we found that LIMK1 had stronger correlations with M2 macrophages and Treg cells, according to the analysis of cell surface markers. Among these markers, FOXP3 and CD163 showed the highest correlation. FOXP3 is a crucial surface protein in Treg cells, which inhibits cytotoxic T cells from attacking tumor cells[32]. Research [33] showed that macrophages with high expression of CD163 often predicted poor survival prognosis in a variety of tumors. Inspired by immune-related genes, we hypothesized that immune checkpoint genes are related to LIMK1 expression levels. Thus, these results indicate that LIMK1 may be able to regulate the tumor immune microenvironment in some ways. Although more experiments are needed to confirm these speculations, the results suggest that LIMK1 has a significant relationship with immune cell infiltration in CRC.

However, there are several limitations to our study. First, we only used the online shared database to analyze the expression of LIMK1 and clinicopathological features of CRC. We must admit that there are differences in chip consistency in the database. It is important to verify the results using more clinical data. And Zang et al. showed that imbalanced LIMK1 and LIMK2 expression led to CRC progression and metastasis[34]. Thus, it is appropriate to think LIMK1 and LIMK2 as common detection and research targets in future studies of the LIMK family. Second, the relationship between LIMK1 and tumor-infiltrating cells needs to be further confirmed by experiments in vivo or in vitro.

Taken together, we proved that LIMK1 was upregulated in CRC, and its upregulation trend was closely related to tumor lymph node metastasis and pathological staging in this study. Moreover, we verified the potential relationship between LIMK1 and tumor-infiltrating lymphocytes in CRC for the first time. This indicates that LIMK1 is likely to be a powerful indicator for the diagnosis and treatment of CRC.

# Conclusions

LIMK1 is highly expressed in colorectal cancer, and is closely related to the clinical features and prognosis of patients. In addition, LIMK1 is a promising prognostic indicator that may regulate tumor progression by affecting tumor immune microenvironment in CRC.

## Materials & Methods

# TCGA and GEO

The raw gene transcriptome data of LIMK1 and the clinical data of participants were downloaded from the TCGA and GEO website[35, 36]. The groups without a normal control group were excluded, and the rest were included in the statistical analysis. For follow-up studies, we converted the downloaded gene expression data into TPM format and ID conversion. The processed data were then analyzed using “limma” and “ggplot2” in R software.

# Tumor immune estimation resource2.0 (TIMER2.0) database

TIMER2.0, an online resource that provides comprehensive analysis and visualization functions of tumor-infiltrating immune cells[37]. The efficacy of tumors and immunotherapy is largely influenced by the composition and abundance of immune cells in the tumor microenvironment. TIMER2.0 allows users to select any gene of interest and visualize the correlation of its expression with immune infiltration level in diverse cancer types. In this study, we analyzed the correction of LIMK1 expression and various immune cells in CRC.

# The human protein atlas (HPA)

The expression of proteins in cells, normal tissues, and cancerous tissues is shown in HPA[38]. In this study, we compared the protein expression of LIMK1 between CRC tumorous tissue and normal adjacent tissue by HPA.

# Protein-protein interaction (PPI) networks and functional enrichment analysis

STRING (version 11.5) is available online and is user-friendly[39]. We searched for co-expressed genes of LIMK1 by STRING and constructed PPI networks using the top 10 genes by interaction scores. Gene Ontology (GO) term enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of the genes obtained by screening were analyzed by “ClusterProfiler” package and “ggplot2” package in R software.

# Correlation analysis of immune cell markers in GEPIA2

The online database Gene Expression Profiling Interactive Analysis 2 (GEPIA2) is an online website with multiple functions, such as genetic difference analysis, survival prognosis, and correlation analysis in multiple cancer types. We found few gene markers that are currently widely recognized in immune cells.

The correlation between LIMK1 and immune cell markers was analyzed by comparing their expression in the tumor tissues. The correlation coefficient was analyzed using Spearman's method.

# Cell lines culture

The CRC cell lines SW480, LOVO, HCT116, DLD-1, SW620, and CRC normal epithelial cell line NCM460 were obtained from the Laboratory of General Surgery, Tianjin Medical University General Hospital (Tianjin, China). These cell lines were cultured in RPMI-1640 medium (Gibco) containing 10% FBS (Hyclone) and 1% penicillin streptomycin (Gibco), and incubated at 37°C with 5% $CO_2$. Cell lines were tested for mycoplasma (PCR Mycoplasma, Venor GeM Mycoplasma Detection Kit, Sigma-Aldrich), and negative results were obtained.

# RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

This study was approved by the Ethics Committee of the Tianjin Medical University General Hospital Ethical Committee (2021-WZ-203). All participants signed an informed consent form. Tissue samples were stored in a refrigerator at -80 ℃. Total RNA from CRC cell lines and tissue samples was extracted with TRIzol reagent (Vazyme) according to the manufacturer's instructions. The concentration of RNA was measured using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, Inc.). The 260/280 ratios of RNA from 1.7-2.0 was reversely transcribed using the FastQuant RT Super mix kit (Tiangen Biotech, Co., Ltd.). Subsequently, qRT-PCR was performed using SYBR Green qPCR Master Mix (Bimake Biotechnology). mRNA expression was quantified using the $2^{-\Delta\Delta CT}$ method. GAPDH was used to normalize LIMK1 expression. The primer sequences were as follows: LIMK1 forward, 5′-TTGCCAAGGACATCGCATCAGG-3′ and reverse, 5′- CGAAGTCAGCCACCACCACATT-3′; GAPDH forward, 5′- TGGCACCGTCAAGGCTGAGAA-3′ and reverse,

5′- TGGTGAAGACGCCAGTGGACTC-3′.

# Statistical analyses

All statistical analyses were performed and visualized using R software (4.1.0) and R packages mentioned above. Comparisons between tumor tissues and normal tissues were performed using the paired t-test or Mann-Whitney U-test depending on data distribution. We plotted ROC curves and calculated the ROC curve using the R package(pROC). Survival analysis was performed using the survival package.

# Abbreviations

| | |
|---|---|
| CRC | Colorectal cancer |
| LIMK1 | LIM Domain Kinase 1 |
| TCGA | The Cancer Genome Atlas |
| GEO | Gene expression omnibus |
| GEPIA2 | Gene expression profiling interactive analysis 2 |
| qRT-PCR | Quantitative real-time-polymerase chain reaction |
| ROC | Receiver operating characteristic |
| DSS | Disease specific survival |
| KEGG | Kyoto encyclopedia of genes and genomes |
| PPI | Protein-Protein Interaction |

# Declarations


### Acknowledgements

Not applicable


### Authors' contributions

Xin Liu and Qiang Song analyzed LIMK1 expression data of CRC from the TCGA and GEO databases. Daohan Wang and Yubiao Liu conducted experimental validation. Zhixiang Zhang and Weihua Fu were major contributors in writing the manuscript. All authors read and approved the final manuscript.


### Funding

This study was supported by Tianjin Municipal Bureau of Public Health (Tianjin Health Institution Key Projects [grant numbers 15KG148]).


### Availability of data and materials

The datasets used or analyzed during the current study can be acquired from the corresponding author upon reasonable request.

### Ethics approval and consent to participate

This study was approved bythe Ethics Review Committee of Tianjin Medical University General Hospital.

### Consent for publication

Not applicable

### Competing interests

The authors declare that they have no competing interests.

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# Figures

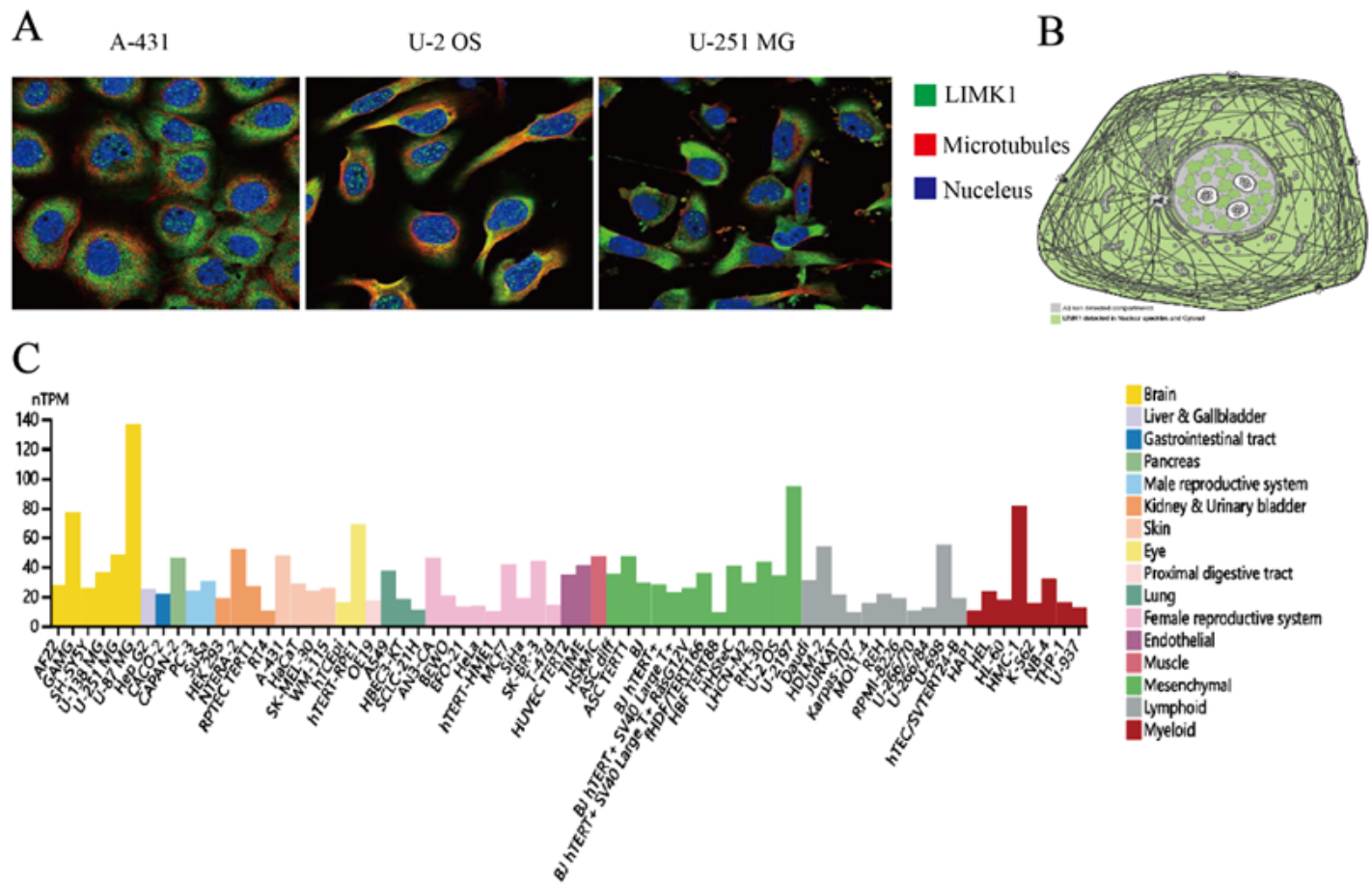



Figure 1

LIMK1 location and expression in different level.

(A) The subcellular distribution of LIMK1, nucleus and microtubules of A-431, U-2 OS, and U-251 MG cells as obtained from the HPA database. (B) LIMK1 expression pattern diagram. (C) The expression of LIMK1 in cell lines. (D) mRNA expression differences of LIMK1 in Pan-cancer. Green dots represents normal adjacent tissue and red dots represented tumor tissue. The tumor abbreviation in red meant that LIMK1 was up-regulated in tumor tissue. The tumor abbreviation in green meant opposite. Abbreviations: ACC, Adrenocortical carcinoma; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, Colon adenocarcinoma; COADREAD, Colon adenocarcinoma/Rectum adenocarcinoma Esophageal carcinoma; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA,Esophageal carcinoma; GBM,Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, Kidney Chromophobe; KIPAN, Pan-kidney cohort (KICH+KIRC+KIRP); KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma, LAML, Acute Myeloid Leukemia; LGG, Brain Lower Grade Glioma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; MESO, Mesothelioma; OV, Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin Cutaneous Melanoma; STAD, Stomach adenocarcinoma; STES, Stomach and Esophageal carcinoma; TGCT , Testicular Germ Cell Tumors; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma; UCS, Uterine Carcinosarcoma; UVM, Uveal Melanoma.

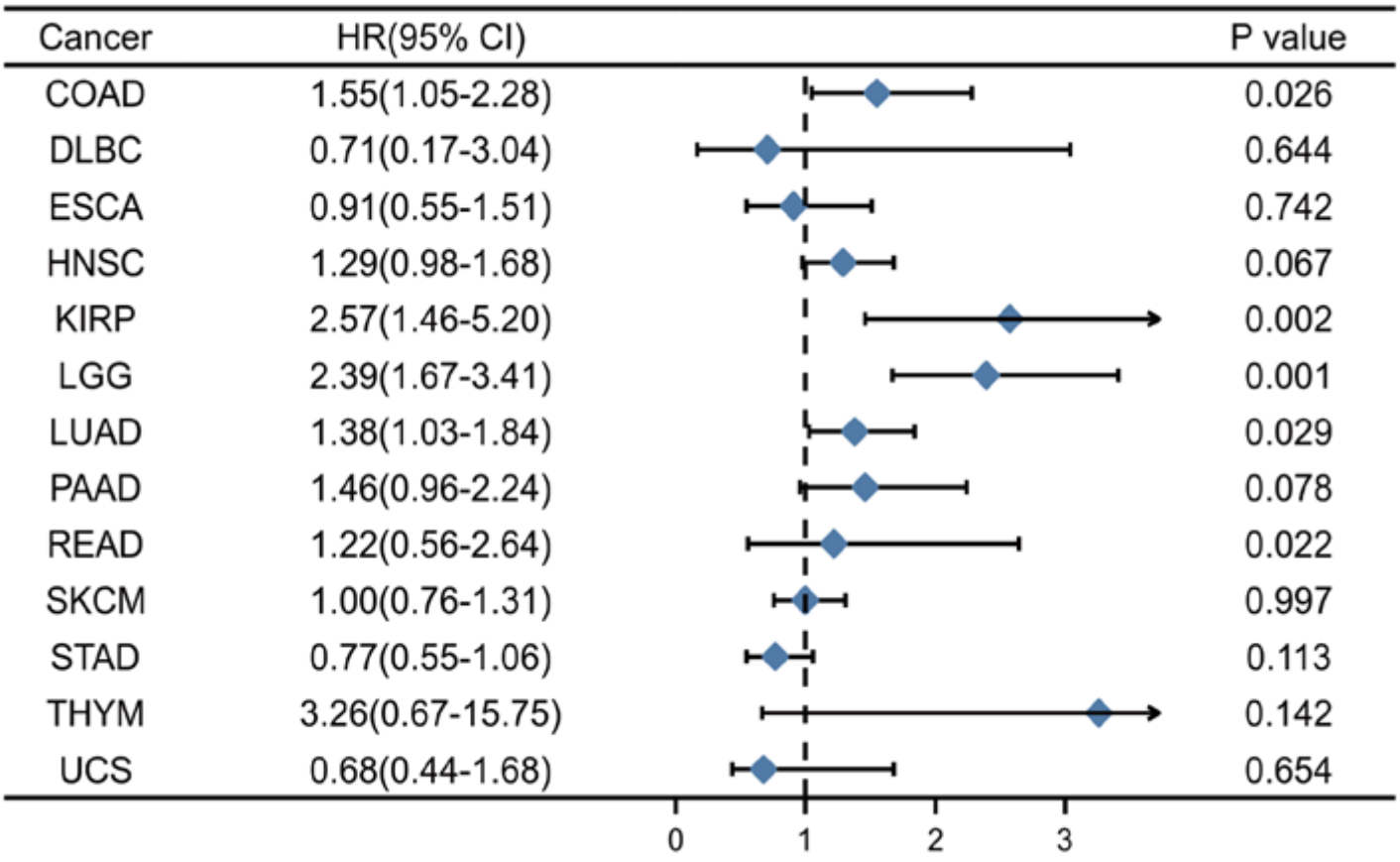



**Figure 2**

**The relationship between LIMK1 expression level and overall survival time across tumors which showed differential expression of LIMK1 compared to normal tissues.**

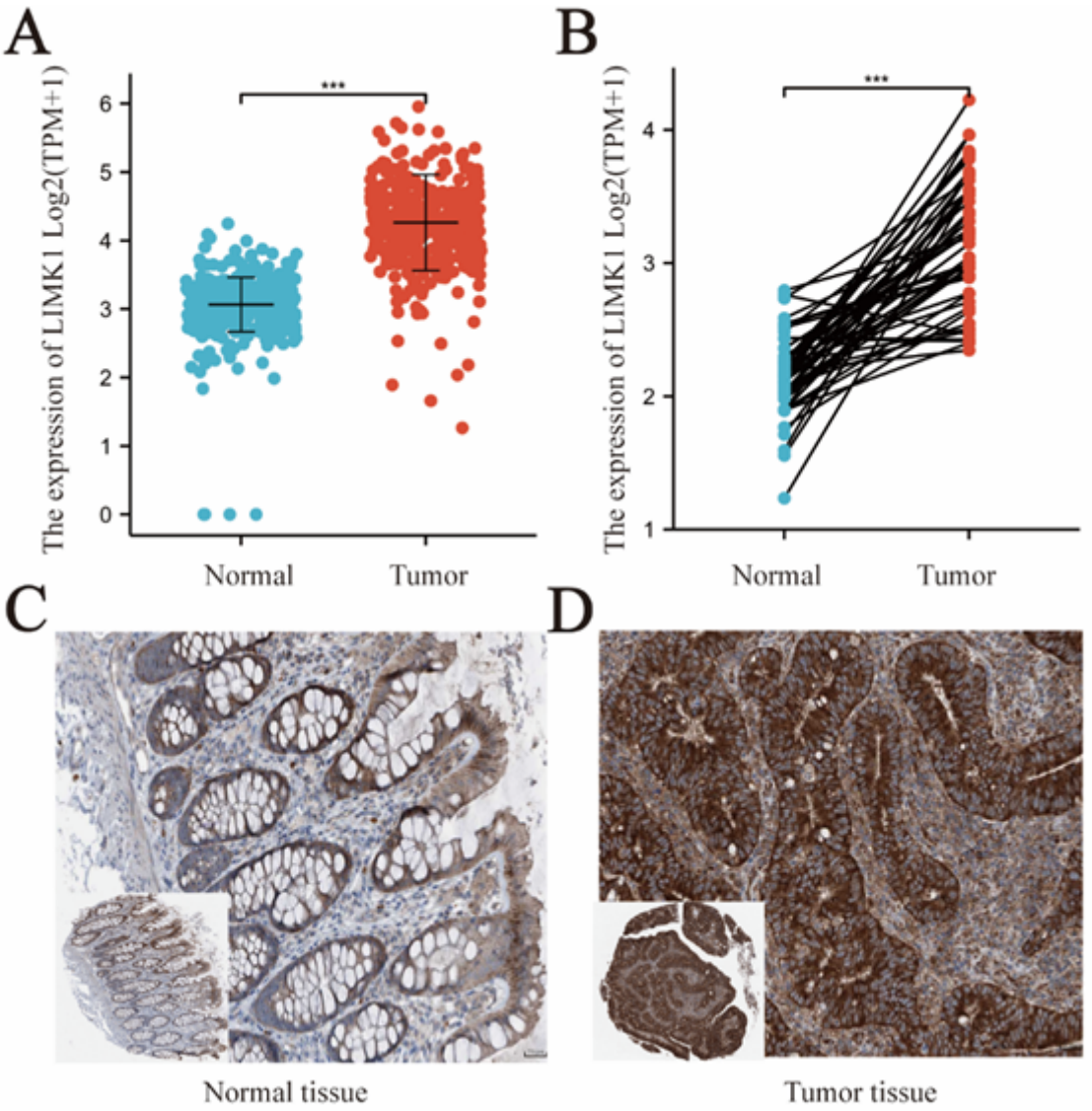



**Figure 3**

**The expression of LIMK1 mRNA and protein in CRC.**

(A) In unpaired samples, mRNA expression levels of LIMK1 was significantly increased in tumor tissues compared to normal tissues (*P* = 1.08e-03). (B) In paired samples, mRNA expression levels of LIMK1 was significantly increased in tumor tissues compared to normal tissues (*P* = 1.12e-03). (C-D) The protein expression level of LIMK1 in normal and tumor tissues on the basis of TPA.

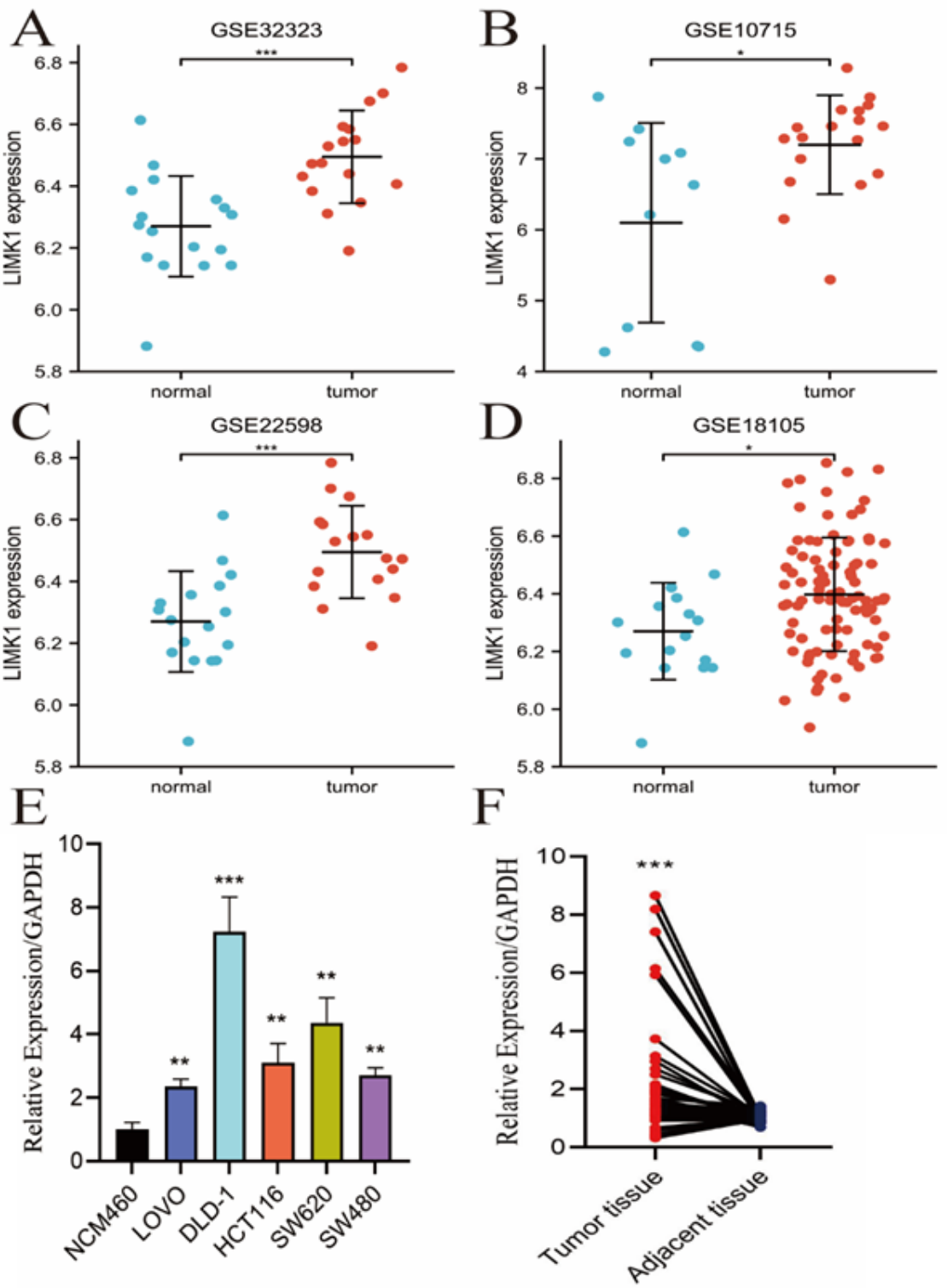
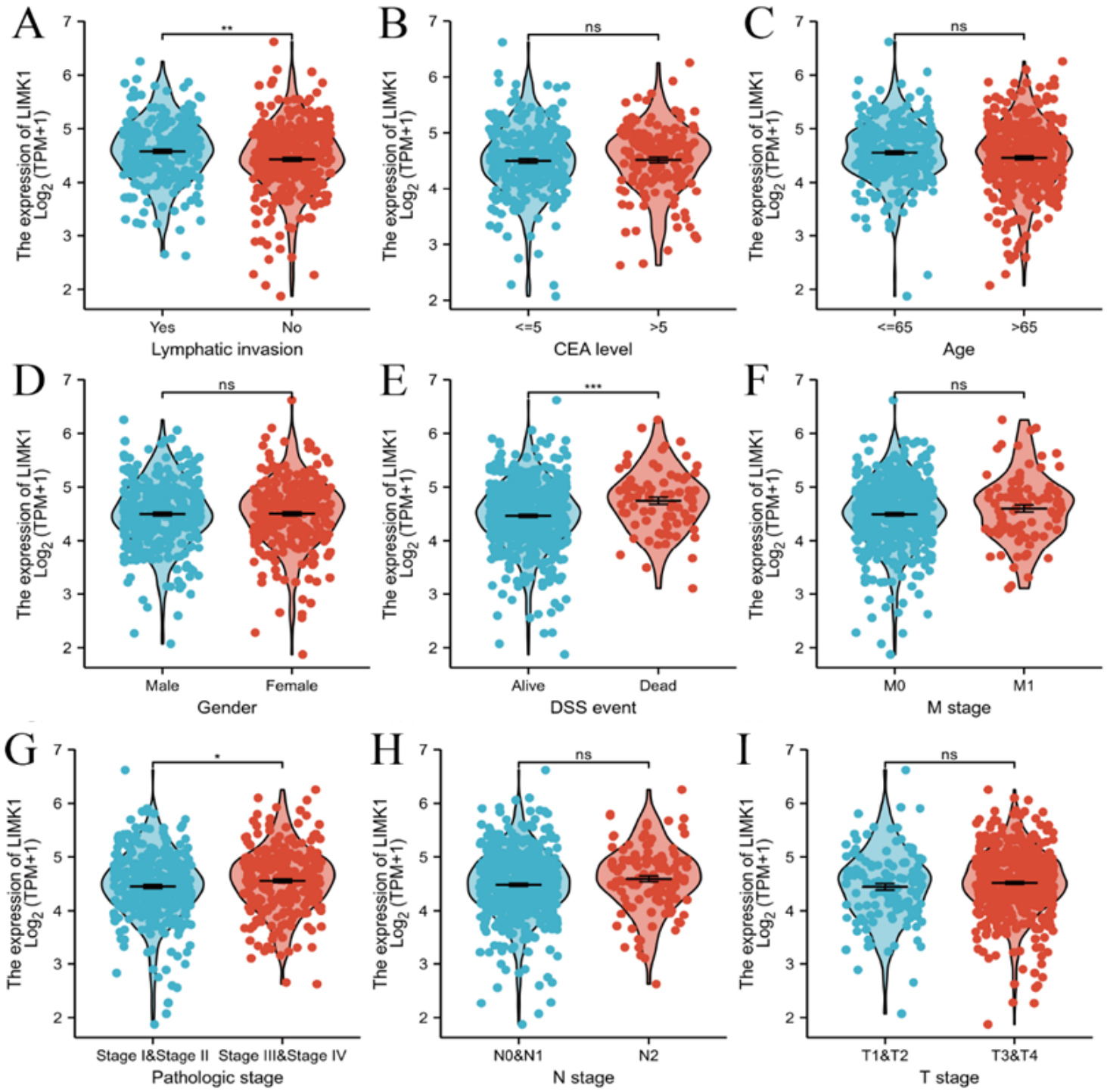



**Figure 4**

**The external verification of GEO databases.**

(A-D) LIMK1 expression was verified higher than normal tissues in GSE 10715, GSE 18105, GSE 22598, GSE 32323. (E) The expression in CRC cell lines (SW480, LOVO, HCT116, DLD-1 and SW620) and normal

epithelial cell line (NCM460). (F) LIMK1 expression level was upregulated in CRC tissues (n=46). (* *P*< 0.05, ** *P*<0.01, *** *P*< 0.001)

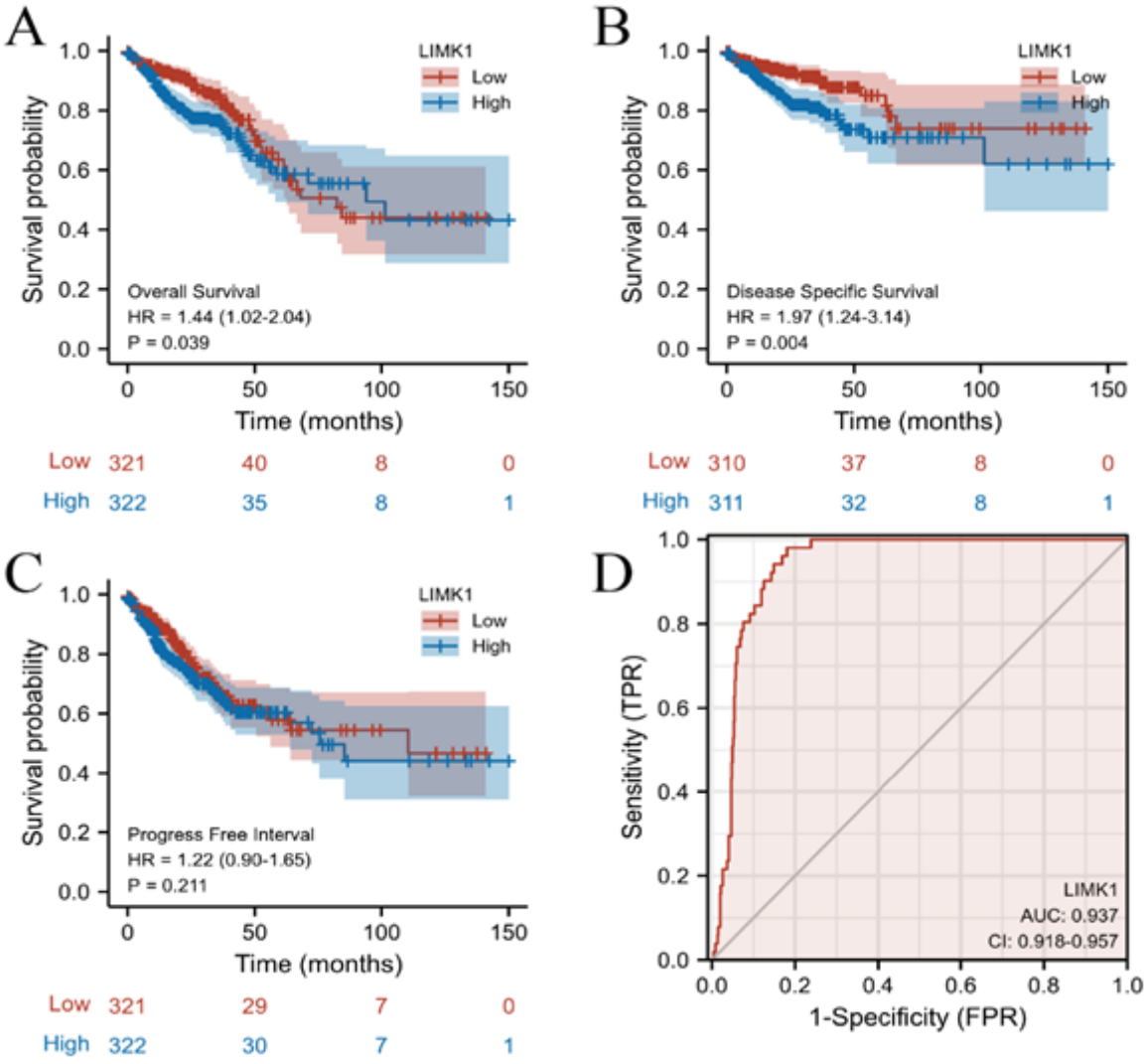



## Figure 5

### The relationship of LIMK1 mRNA and clinicopathological features of CRC.

Increased LIMK1 expression was highly associated with lymphatic invasion, high TNM stage and DSS (disease-specific survival). No statistical difference were found in other features. (ns, no significance, *P <

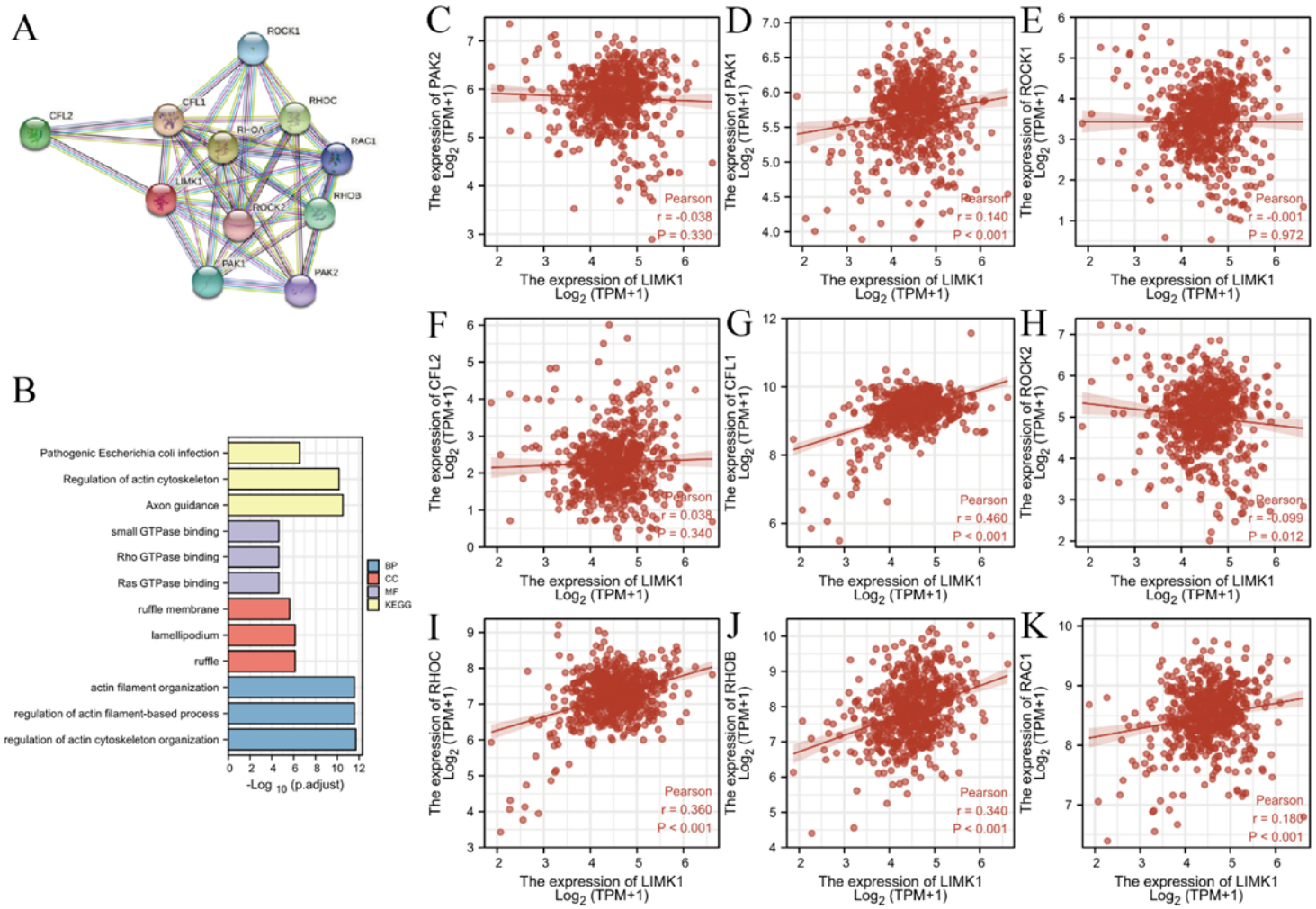
0.05, ** *P*< 0.01, *** *P* < 0.001).




## Figure 6

### Kaplan-Meier and ROC curves of LIMK1.

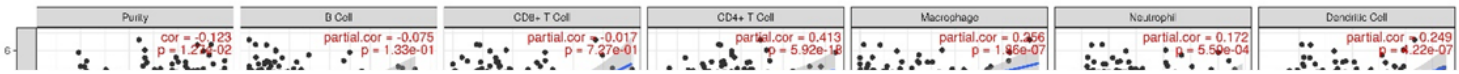
(A) The AUC value of LIMK1 in CRC was 0.937 (95% CI: 0.918–0.957). Under the best cut-off value (4.009), the sensitivity and specificity was 98% and 81.9%, respectively. (B-D) Kaplan-Meier curves showed that CRC patients with lower LIMK1 expression level had longer OS (*P* = 3.90e-02) and DSS (*P* = 4.00e-03) than patients with higher expression. There was no difference in PFI (*P* = 0.211).




## Figure 7

**The PPI networks and functional annotations of LIMK1.**

(A) LIMK1 and its co-expression genes. (B) The functional enrichment analyses of LIMK1 and its co-expression genes. The lamellipodium, ruffle and cell leading edge represented cellular component. The rho GTPase binding, ras GTPase binding and protein serine/threonine kinase represented molecular function. The actin filament–based process, regulation of actin cytoskeleton organization, and actin filament organization represented biological process. And the last three indicators showed KEGG analyses. (C-K) The specific correlation of LIMK1 and its co-expression genes in CRC. CFL1 (r=0.46), RHOB (r=0.36), RHOC (r=0.36) had the most significant correlation with LIMK1.




## Figure 8

### The correlation of LIMK1 with immune cell infiltration in CRC.

LIMK1 was correlated with the expression of multiple immune cells in the tumor microenvironment, among which $CD4^+$ cells macrophages and dendritic cells have the highest correlation.

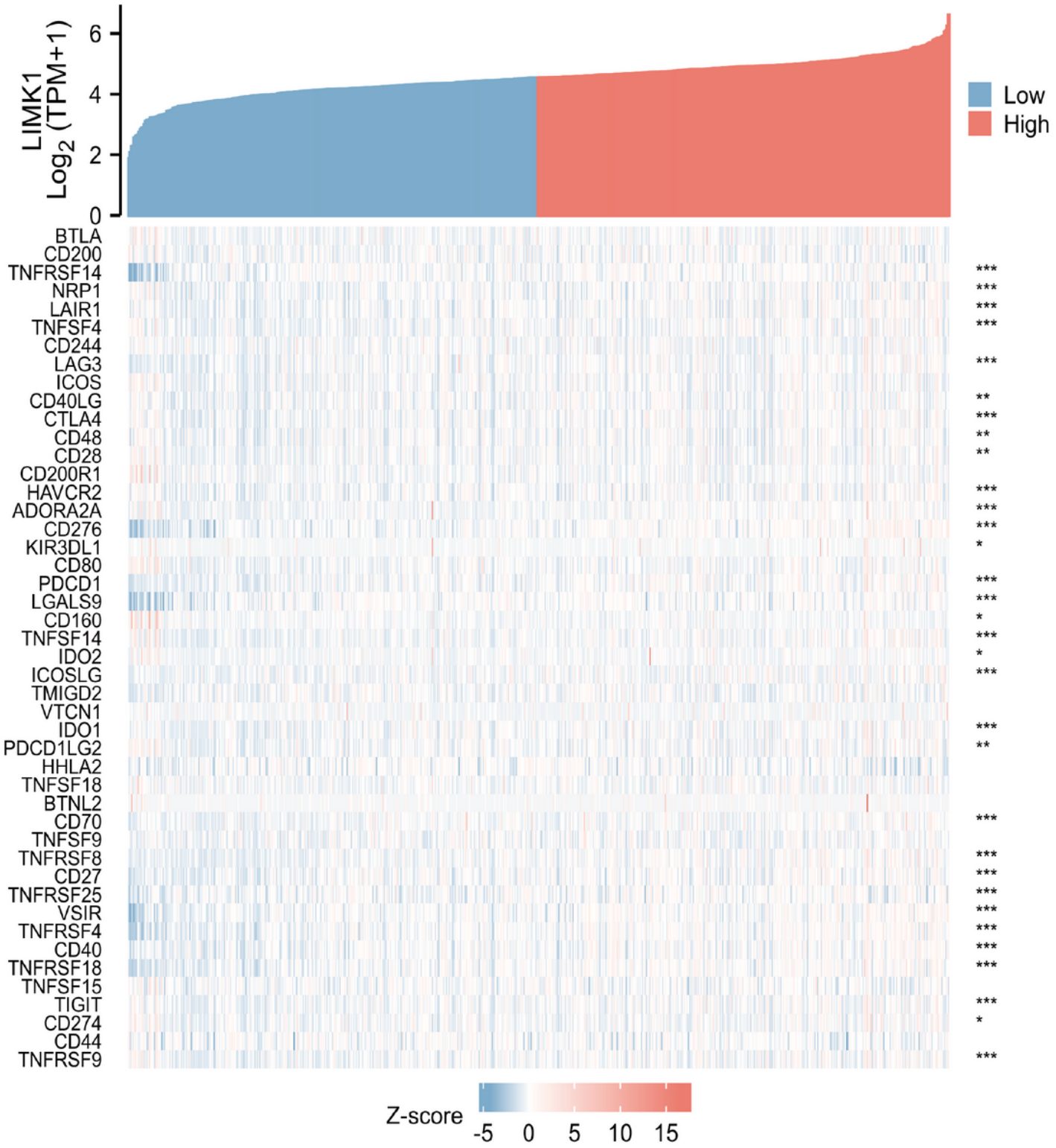



**Figure 9**

**The correlation of LIMK1 with fifty immune checkpoint genes in CRC.** (blank, no significance, * *P*< 0.05, ** *P* < 0.01, *** *P*< 0.001).

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.TheexpressionofLIMK1inpairedCRCpatientsfromTCGA.xlsx
- Additionalfile2.TheexpressionofLIMK1inunpairedCRCpatientsfromTCGA.xlsx
- Additionalfile3.qRTPCRdataofCRCcelllines.xlsx
- Additionalfile4.qRTPCRdataoftissuesamples.xlsx
- Additionalfile5.ThecorrelationshipbetweenLIMK1andimmunecheckpoint.xlsx