Pan-cancer analyses reveal the regulation and clinical outcome association of PCLAF in human tumors

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Abstract. Studies have shown that PCNA clamp associated factor (PCLAF) plays a paramount role in a variety of cancers; however, the expression profile and the specific molecular mechanism of PCLAF in cancer remains unclear, as is its value in the human pan-cancer analysis. Based on the publicly available datasets of The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO), a comprehensive analysis of the probable carcinogenic effects of the *PCLAF* gene was performed in 33 human cancers. It was found that *PCLAF* is highly expressed in cancer tissues compared with normal tissues, and is significantly correlated with poor prognosis. We found that the eight tumors with significantly high PCLAF expression presented with decreased DNA methylation levels of PCLAF, including cholangiocarcinoma (CHOL), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), glioblastoma multiforme (GBM), pheochromocytoma and paraganglioma (PCPG), sarcoma (SARC), testicular germ cell tumor (TGCT), stomach adenocarcinoma (STAD), and uterine corpus endometrial carcinoma (UCEC). The expression of PCLAF was found to be positively correlated with activated CD4 T cells (Act CD4) and type 2 T helper (Th2) cells, suggesting that PCLAF may play a particular role in tumor immune infiltration. In addition, the functional mechanism of PCLAF also involves the mitotic cell cycle process, cell division, and DNA replication. Our first pan-cancer study provides a relatively extensive understanding of the carcinogenic effects of PCLAF in miscellaneous tumors.

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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; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; 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; MM, multiple myeloma; MCC, Merkel cell carcinoma; 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; TGCT, testicular germ cell tumor; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; TIMER2, Tumor Immune Estimation Resource; CAN, copy number alteration; HCC, hepatocellular carcinoma; OS, overall survival; RFS, relapse-free survival; DFS, disease-free survival; DMFS, distant metastasis-free survival; FP, first progression; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; TILs, tumor infiltrating lymphocytes; TME, tumor microenvironment

Key words: PCLAF, cancer, prognostic biomarker, methylation, immune infiltration, cell cycle

Introduction

Pan-cancer analysis can elucidate the common characteristics and heterogeneity of human malignancies by analyzing the molecular abnormalities of various types of cancers (1). The publicly funded The Cancer Genome Atlas (TCGA) project and the available Gene Expression Omnibus (GEO) database contain functional genomic datasets of different tumors, thus conducting pan-cancer analysis (2-4). Therefore, pan-cancer analysis is beneficial to the advancement of combination therapies and individualized therapies to apply treatment to various cancer models.

PCNA clamp associated factor (PCLAF), also known as KIAA0101, was initially identified by a yeast two-hybrid (5). PCLAF interacts with proliferating cell nuclear antigen (PCNA) through Lys15 and Lys24 sites to recruit DNA replication polymerase (6). When DNA is damaged, PCLAF regulates the conversion of DNA replication polymerase into translation synthesis polymerase, thereby bypassing the diseased area and continuing DNA replication (7). Research has demonstrated

that PCLAF can promote the proliferation of undifferentiated thyroid cancer and cervical cancer cell lines, DNA synthesis and cell viability of pancreatic cancer and adrenal cancer cell lines, and reduce the number of G0/G1 cells in adrenocortical cancer cell lines (8-13). Up-regulation of PCLAF can accelerate the repair of UV-induced DNA damage and prevent cell death. In contrast, reduction in PCLAF expression can inhibit DNA replication (7,14-16). PCLAF is overexpressed in a myriad of human malignancies and is associated with poor patient prognosis (17-19).

In the present study, the TCGA project and the GEO database were utilized to perform a pan-cancer analysis of PCLAF for the first time. We also incorporated factors such as gene expression, survival status, methylation status, genetic changes, immune infiltration, and related cellular pathways to explore the potential molecular mechanisms of PCLAF in the onset or clinical prognosis of different types of tumors.

Materials and methods

Gene expression analysis. The ONCOMINE database (www.oncomine.org) was referred to in order to examine the expression levels of *PCLAF* mRNA in distinct types of cancers [(P=0.001, 1.5-fold change were set as significance thresholds]. Using the Gene_DE module of the Tumor Immune Estimation Resource, version 2 (TIMER2) website (http://timer.cistrome.org/) database, we examined the expression discrepancy between *PCLAF* tumors and adjacent normal tissues in the TCGA project. For tumors lacking paired normal tissues in the TIMER2 database [for example, TCGA-GBM (glioblastoma multiforme), TCGA-LAML (acute myeloid leukemia)], the 'Expression Analysis-Box Plots' module of Gene Expression Profile Interactive Analysis (GEPIA2) version 2 webserver (http://gepia2.cancer-pku.cn/analysis) (20) was performed to gain box plots of the *PCLAF* expression profile between the tumor tissue and corresponding normal tissue from the Genotype-Tissue Expression (GTEx) database, and the hypothetical value was set to cut-off=0.01, log2FC (fold-change) cutoff=1, and Match TCGA standard and GTEx data. In addition, we obtained a violin chart of *PCLAF* expression in all TCGA tumors at different pathological stages (stage I, II, III, IV) through the pathological staging diagram module of GEPIA2. The box or violin chart used log2 [TPM (transcripts per million) +1] transformed expression data.

UALCAN portal (http://ualcan.path.uab.edu/analysis-prot.html), a database for analyzing cancer omics data, allowed us to perform protein expression analysis on The National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC) dataset (21). Here, we entered PCLAF to explore total protein expression levels in primary tumors and normal tissues.

Survival prognosis analysis. The Survival Map module in GEPIA2 was used to obtain overall survival (OS) and disease-free survival (DFS) saliency map data of *PCLAF* in all TCGA tumors. The cut-off high value (50%) and cut-off low value (50%) were applied to the expression thresholds for dividing the high and low expression cohorts. The 'Survival Analysis' module of GEPIA2 was used to obtain the P-value by log-rank test. Kaplan-Meier Plotter (http://kmplot.com/analysis/) is a powerful online tool capable of assessing the impact of 54,000 genes in 21 cancer types concerning survival (22). We analyzed the relationship of *PCLAF* expression with OS, distant metastasis-free survival (DMFS), relapse-free survival (RFS), disease-specific survival (DSS), first progression (FP), progression-free survival (PFS) for breast cancer, liver cancer, and lung cancer. Hazard ratios with a 95% confidence interval (CI) and log-rank P-values were calculated.

Genetic alterations. To study the genetic changes of the *PCLAF* gene in the pan-cancer cohort, we logged into the cBioPortal website (https://www.cbioportal.org/) (23,24). We selected the 'TCGA Pan-Cancer Atlas Studies' in the 'Quick select' section and entered 'PCLAF' for queries of the genetic alteration characteristics of *PCLAF*. The Cancer Types Summary module was used to observe the mutation frequency, mutation type, and copy number change (CNA) results of all TCGA tumors. The mutation site information of PCLAF was displayed in the protein structure diagram or three-dimensional structure through the mutation module.

Immune infiltration analysis. The immune gene module in the TIMER2 database (http://timer.cistrome.org/) was used to explore the relationship between PCLAF expression and immune infiltration in all TCGA tumors (25-27). The TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, XCELL, MCPCOUNTER and EPIC algorithms were performed for immune infiltration estimations. The Spearman rank correlation test was used to obtained the P-value and the partial correlation (cor) with purity adjustment. The data are visualized as heat maps and scatter plots.

Regulatory networks of transcription factors (TFs). To study the epigenetic alterations of PCLAF, TFs with binding ability to the PCLAF promoter were anticipated using Harmonizom (https://maayanlab.cloud/Harmonizome) (28), including CHEA Transcription Factor Targets. GSCA Lite (http://bioinfo.life.hust.edu.cn/web/ GSCA Lite/) is an integrated genomic, and immunogenomic web-based platform for gene set cancer research (29).

Tumor-Immune System Interaction Database (TISIDB) and Tumor Immune Single Cell Hub Database (TISCH). TISIDB is an online database of tumor-immune system interactions (30). In the present study, we used TISIDB to determine the expression of PCLAF and tumor-infiltrating lymphocytes (TILs) in human cancers. Based on the gene expression profile, gene set variation analysis was used to infer the relative abundance of TILs. Spearman test was used to determine the correlation between PCLAF and TILs. The Tumor Immune Single-Cell Center (TISCH, http://tisch.comp-genomics.org) is an online database focusing on the tumor microenvironment (TME). It collects 76 tumor datasets for 27 types of cancer, including single-cell transcriptome profiles of nearly 2 million cells (31).

PCLAF-related gene enrichment analysis. We first searched the STRING website (https://string-db.org/) using the query of a single protein name ('PCLAF') and organism ('*Homo sapiens*'). Subsequently, we set the following main parameters:

minimum required interaction score ['Low confidence (0.150)'], the meaning of network edges ('evidence'), max number of interactors to show ('no more than 50 interactors' in 1st shell). Finally, the available determined PCLAF-binding proteins were obtained. We used the 'Similar Gene Detection' module of GEPIA2 to receive the top 100 PCLAF-correlated targeting genes based on the datasets of all TCGA tumors and normal tissues. We also applied the 'correlation analysis' module of GEPIA2 to perform a pairwise gene Pearson correlation analysis of PCLAF and selected genes. The log2 TPM was applied for the dot plot. The P-value and the correlation coefficient (R) are indicated. Moreover, we used the 'Gene_Corr' module of TIMER2 (http://timer.cistrome.org/) to supply the heatmap data of the selected genes, which contains the partial correlation (cor) and P-value in the purity-adjusted Spearman's rank correlation test. Metascape (http://metascape.org.) is an effective and efficient tool to comprehensively analyze and interpret OMICs-based studies (32).

Cell culture and lentivirus-mediated silencing of PCLAF. The HepG2 cell line (liver cancer cells) was purchased from the Chinese Academy of Sciences, which was cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gemini Bio Products) and was incubated at 37˚C in a humid incubator with air containing 5% CO_2. Lentivirus, including complementary oligonucleotide sequences, target sequences were as follows: 5'-CATGGTGCGGACTAA AGCA-3', were performed and synthesized by Genomeditech. At 72 h post-transfection, 2 μg/ml puromycin (cat. no. ST551; Beyotime Institute of Biotechnology) was used to select the stably transfected cell lines.

Cell viability analysis. Cell Counting Kit-8 (CCK-8) (Yeasen) was used to analyze cell viability. The cells were seeded in 96-well plates at a density of $5x10^3$ per well and cultured for 4 days. After cells were adherent to the bottom of wells, the CCK-8 assay was then performed according to the manufacturer's instructions. The absorbance of each well was determined with a microplate reader set at 450 and 630 nm

EdU incorporation test. For flow cytometry analysis of the proliferating cells, a Cell-Light EdU Apollo 488 *In vitro* Flow Cytometry Kit (RiboBio) was used to examine EdU-positive cells according to the manufacturer's protocol. The fluorescence signal at 488 nm was performed with a flow cytometer.

Cell cycle detection. According to the protocol of the Cell Cycle and Apoptosis Analysis kit (C1052), the cells were harvested and stained with propidium iodide (PI). Then the cell cycle was measured by flow cytometry.

Annexin V-FITC/propidium iodide (PI) flow cytometry. HepG2 cells were plated in a 6-well plate and transfected with shPCLAF after 24 h. After 48 h, an Annexin V-FITC kit (BD Biosciences) was used to evaluate cell apoptosis according the instructions of the manufacturer.

Western blotting. Western blotting was implemented as mentioned previously (33). Primary antibodies against PCLAF (cat. no. 81533S; 1:1,000), GAPDH (cat. no. 2118s; 1:2,000), β-actin (cat. no. 3700S; 1:1,000), cyclin D1 (cat. no. 2978s; 1:1,000), cyclin A2 (cat. no. 4656S; 1:1,000), cyclin B1 (cat. no. 12231S; 1:1,000), cyclin E2 (cat. no. 4132S; 1:1,000), CDK2 (cat. no. 18048S; 1:1m000), CDK6 (cat. no. 13331S; 1:1,000), BAX (cat. no. 14796S; 1:1,000), and Bcl-2 (cat. no. 15071S; 1:1,000) were purchased from Cell Signaling Technology, Inc. The Apoptosis Antibody Sampler Kit (cat. no. 9915T; 1:1,000) was also purchased from Cell Signaling Technology.

Quantitative real-time PCR. We extracted total RNA from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription reactions were performed with a reverse transcription kit (cat. no. RR036A; Takara Bio, Inc.). SYBR Green Master Mix (cat. no. 11198ES03; Yeasen) was used for quantitative real-time PCR (qPCR). Primer sequences were as follows: PCLAF forward, 5'-GGCAAGGAGGAC AAATACGCA-3' and reverse 5'-TGTGCCCACCATGATTCT ATCC-3'; Relative mRNA expression levels were determined with the internal control GAPDH using the $2^{-\Delta\Delta Cq}$ method (34).

Statistical analysis. The results generated by Oncomine are presented by P-values as determined by t-tests, fold-changes, and gene rankings. The Kaplan-Meier method was used to estimate the survival curves. In order to compare survival curves, we used log-rank test to calculate the HR and log-rank P-values of Kaplan-Meier Plotter and GEPIA. The univariate Cox regression model was used to calculate the HR and Cox P-values of the prognostic scan. Spearman correlation was used to evaluate the correlation of gene expression. The results are expressed as the mean ± standard error of the mean (SEM). A P-value <0.05 was considered to indicate a statistically significant difference. Significance is expressed as: *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, as denoted in the figures and figure legends.

Results

mRNA expression level of PCLAF in pan-cancer. The foremost aim of the present investigation was to study the carcinogenic effects of human PCLAF (NM_014736.6for mRNA or NP_055551.1 for protein). First, we conducted an investigation into the PCLAF expression pattern in different cells and non-tumor tissues. As shown in Fig. S1A, combined with the Human Protein Atlas (HPA) dataset (Human Protein Atlas proteinatlas.org) (35,36), the Genotype-Tissue Expression (GTEx) dataset, and The Functional Annotation of the Mammalian Genome 5 (FANTOM5) dataset, PCLAF was found to be predominantly expressed in the 'Thymus', followed by 'Bone metastasis' and 'T cells'. However, PCLAF exhibited low RNA tissue specificity at the tissue level. Furthermore, we evaluated the *PCLAF* RNA expression in cell lines and blood cells in the HPA/Monaco/Schmiedel datasets and found that low RNA specificity also was evident (Fig. S1B and C). To determine the expression level of PCLAF in distinct human tumors and adjacent normal tissues, the *PCLAF* mRNA expression levels were analyzed using the ONCOMINE database (Fig. 1A). The results showed that *PCLAF* expression was outstandingly escalated in most cancer types, such as bladder, brain and central

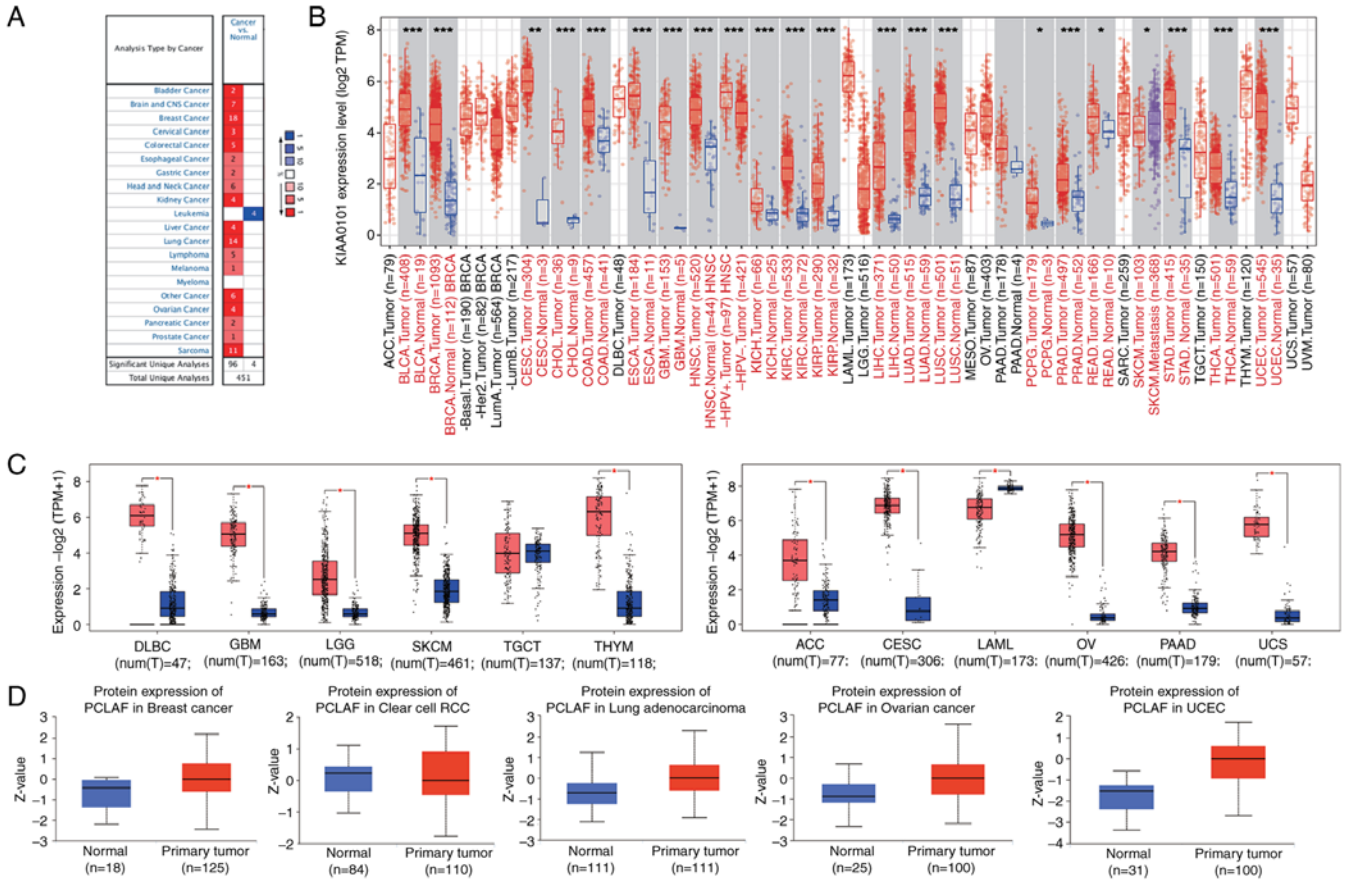

Figure 1. Expression level of the *PCLAF* gene in different tumors and pathological stages. (A) Increased or decreased expression of PCLAF in different cancer tissues, compared with normal tissues in ONCOMINE. The number in each cell is the number of the dataset. (B) Human PCLAF expression levels in different cancer types from TCGA data in TIMER. (C) For the DLBC, GBM, LGG, SKCM, TGCT, THYM, ACC, CESC, LAML, OV, PAAD, and UCS tumor types in the TCGA project, the corresponding normal tissues of the GTEx database were included as controls. The box plot data are provided. (D) Based on the CPTAC dataset, we also analyzed the expression level of PCLAF total protein between normal tissue and primary tissue of breast cancer, ovarian cancer, lung adenocarcinoma, clear cell renal cell carcinoma (RCC) and UCEC (*P<0.05, **P<0.01, ***P<0.001). PCLAF, PCNA clamp associated factor, also known as KIAA0101.

nervous system (CNS), breast, cervical, colorectal, esophageal, gastric, head and neck, kidney, liver, lung, lymphoma, melanoma, ovarian, pancreatic cancer, prostate cancer, as well as other cancers. At the same time, low *PCLAF* expression was only found in one leukemia dataset. To further seek out the expression level of *PCLAF* in pan-cancer, we used the TIMER database to identify the RNA sequencing data in TCGA. The differential expression of PCLAF in tumor and adjacent normal tissues is shown in Fig. 1B. PCLAF expression was significantly expressed in bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), -HPV+ tumor, kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA), and uterine corpus endometrial carcinoma (UCEC) than the normal tissues. After including the normal tissue of the GTEx dataset as controls, we further evaluated the expression difference of PCLAF between the normal tissues and tumor tissues of lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), glioblastoma multiforme (GBM), brain lower grade glioma (LGG), testicular germ cell tumors (TGCT), skin cutaneous melanoma (SKCM), thymoma (THYM), adrenocortical carcinoma (ACC), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), acute myeloid leukemia ovarian(LAML), serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), and uterine carcinosarcoma (UCS) as shown in Fig. 1C. The CPTAC database results showed that the expression levels of the total PCLAF protein were elevated in primary tissues of breast cancer, ovarian cancer, clear cell renal cell carcinoma, uterine corpus endometrial carcinoma, lung adenocarcinoma, compared with normal tissues (Fig. 1D).

We also used the 'pathological staging diagram module' of GEPIA2 to evaluate the correlation between the expression of PCLAF and the pathological stage of cancer. Intriguingly, we found that the expression of PCLAF increased with the clinical stage from stage I to stage IV, including ACC, KICH, KIRC, KIRP, LIHC, LUAD, LUSC, and PAAD (Fig. S2A).

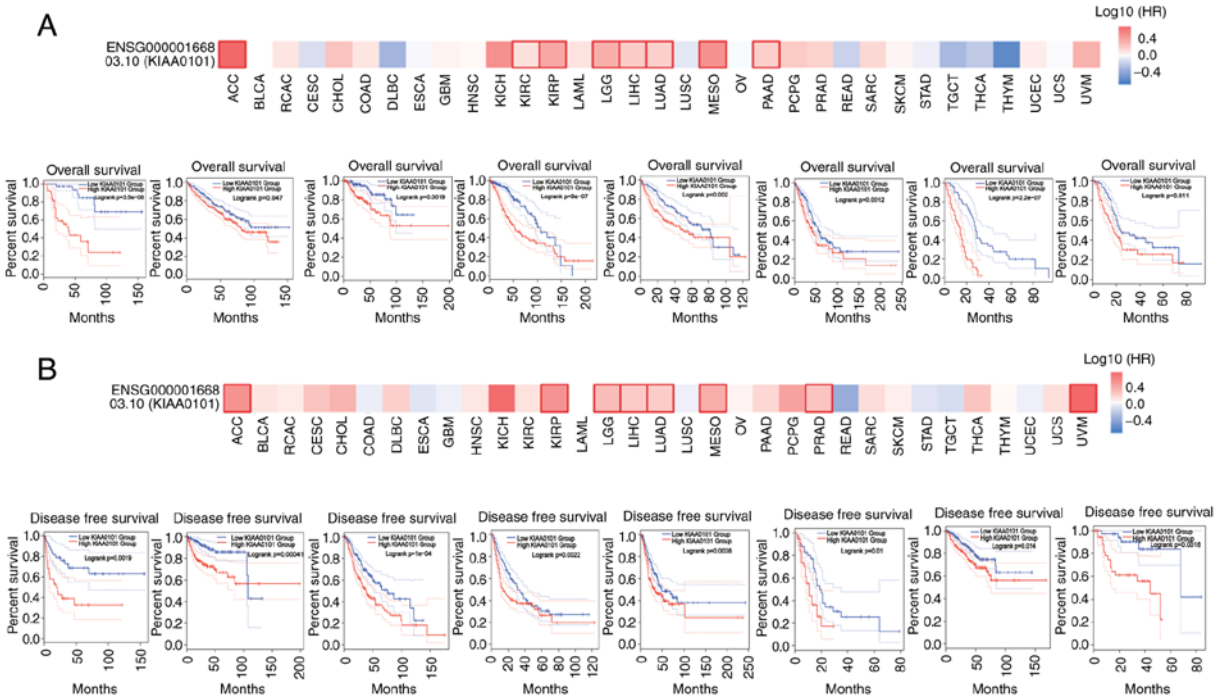

Figure 2. Correlation between *PCLAF* gene expression and survival prognosis of cancers in TCGA. We used the GEPIA2 tool to perform (A) overall survival and (B) disease-free survival analyses of different tumors in TCGA as associated with the *PCLAF* gene expression. The survival map and Kaplan-Meier curves with positive results are provided. PCLAF, PCNA clamp associated factor, also known as KIAA0101.

Multifaceted prognostic value of PCLAF in cancer survival analysis data. First, we divided the cancer samples into high-expression and low-expression groups according to the average expression of PCLAF. We used the TCGA and GEO databases to elucidate the correlation between PCLAF expression and prognosis in tumor patients. As shown in Fig. 2A, high expression of PCLAF was linked to poor OS for cancers of ACC (P=3.5E-06), KIRC (P=0.047), KIRP (P=0.0019), LGG (P=9.0E-07), LIHC (P=0.002), LUAD (P=0.0012), MESO (P=2.2E-07), and PAAD (P=0.011), within the TCGA project. DFS analysis data showed a correlation between increased PCLAF expression and poor DFS for the TCGA cases of ACC (P=0.0019), KIRP (P=0.00041), LGG (P=1.0E-04), LIHC (P=0.0022), LUAD (P=0.0038), MESO (P=0.01), PRAD (P=0.014) and UVM(P=0.0018) (Fig. 2B). The Kaplan-Meier Plotter database was used to further evaluate PCLAF-related survival rates. An increased expression level of PCLAF was associated with poor OS (P=0.0016), distant metastasis-free survival (DMFS) (P=0.00022), and relapse-free survival (RFS) (P<1.0E-16) for breast cancer; OS (P=4.1E-05), disease-specific survival (DSS) (P=0.00011) and RFS (P=8.5E-05) prognosis for liver cancer; and OS (P=1.8E-16), first progression (FP) (P=1.7E-06) and progress-free survival (PFS) (P=0.0057) for lung cancer (Fig. S2B). To better understand the predictive value and possible mechanism of PCLAF expression in LUAD, we used the Kaplan-Meier database to explore the relationship between *PCLAF* mRNA expression and clinical features. Interestingly, PCLAF plays an injurious role in LUAD patients and has the following characteristics. High *PCLAF* expression was significantly correlated with poor OS and PFS in male and female lung cancer patients with adenocarcinoma. High *PCLAF* expression was associated with poor OS and PFS only in stage 1 lung cancer patients in regards to different tumor stages. In American Joint Committee on Cancer (AJCC) N-0 lung cancer patients, *PCLAF* expression was significantly correlated with poorer OS and PFS. In addition, high PCLAF expression was significantly associated with poor OS and PFS in smoking lung cancer patients (Fig. S3). These results suggest that *PCLAF* mRNA expression has prognostic value in lung cancer.

Genetic alteration analysis data. A fundamental cancer analysis of PCLAF in malignant tumors was next performed. In the TCGA pan-cancer group, the most common DNA change was amplification. As shown in Fig. 3A, amplification was mainly distributed in mesothelioma, kidney chromophobe, sarcoma, and prostate adenocarcinoma. Mutation of PCLAF was observed in uterine carcinosarcoma, skin cutaneous melanoma, head and neck squamous cell carcinoma, kidney renal papillary cell carcinoma, cervical squamous cell carcinoma, and brain lower grade glioma patients. In addition, PCLAF deep deletion in malignancies was distributed across stomach adenocarcinoma, esophageal adenocarcinoma, and glioblastoma multiforme. Furthermore, Fig. 3B shows the types, sites, and case number of the PCLAF genetic alteration. The main genetic changes identified in the *PCLAF* gene are missense mutations. The most frequent mutation was F68L/Y alteration, which was detected in 1 case of bladder urothelial carcinoma, 1 case of endometrial carcinoma, and 1 case of endometrial carcinoma (Fig. 3B). We observed the F68L/Y site in the three-dimensional structure of the PCLAF protein (Fig. 3C). We then confirmed the relevance of genetic disorders and PCLAF expression. We found that mutations were not related to RNA expression status (Fig. 3D). In addition, we found

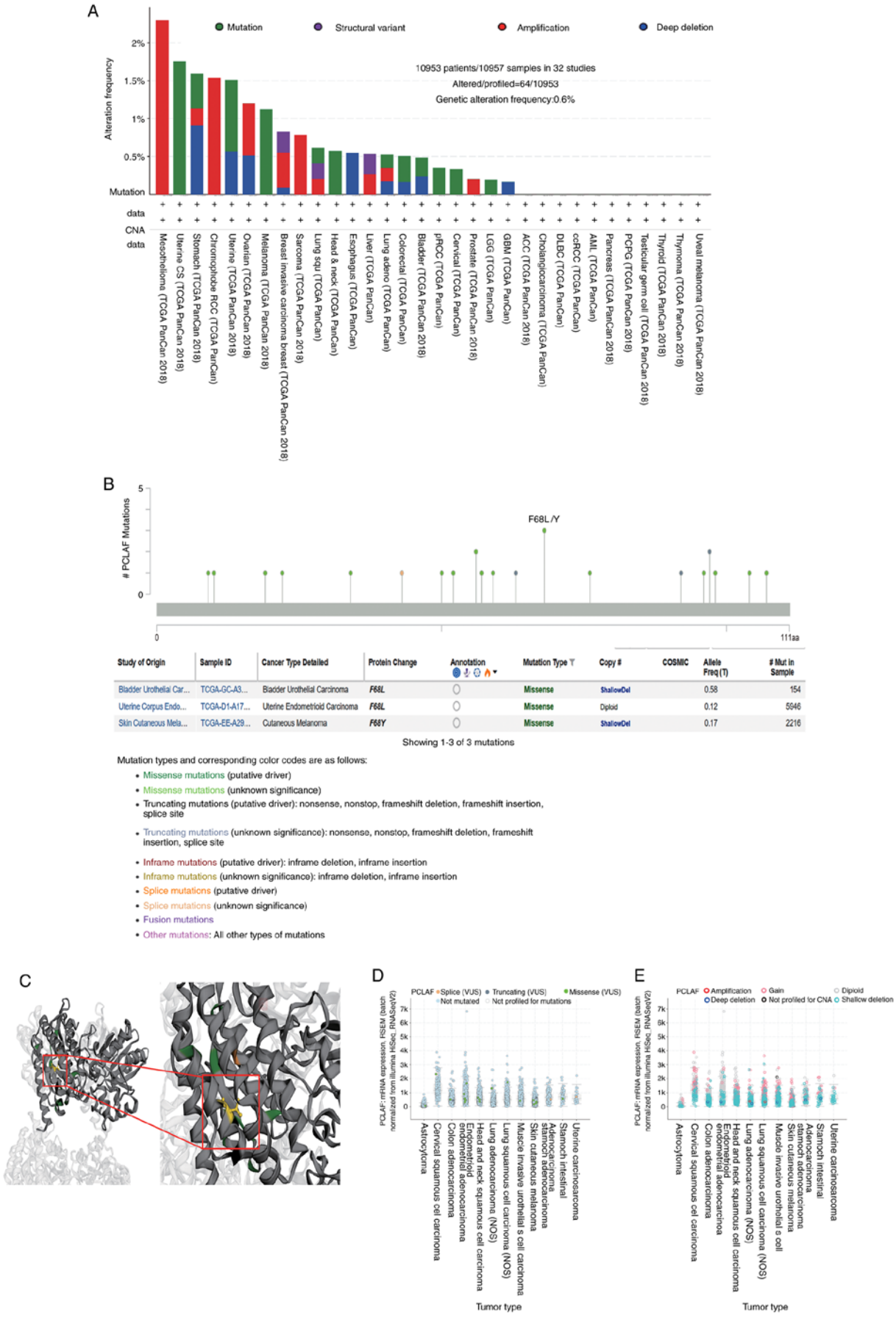

Figure 3. Mutation feature of PCLAF in different tumors of TCGA. We analyzed the mutation features of PCLAF for TCGA tumors using the cBioPortal tool. The alteration frequency with (A) mutation type and (B) mutation site is displayed. (C) The mutation site with the highest alteration frequency (F68LY) in the 3D structure of PCLAF is displayed. (D) Mutations were not relevant to RNA expression. (E) DNA copy variations were not statistically relevant to *PCLAF* RNA expression in most cases. PCLAF, PCNA clamp associated factor, also known as KIAA0101.

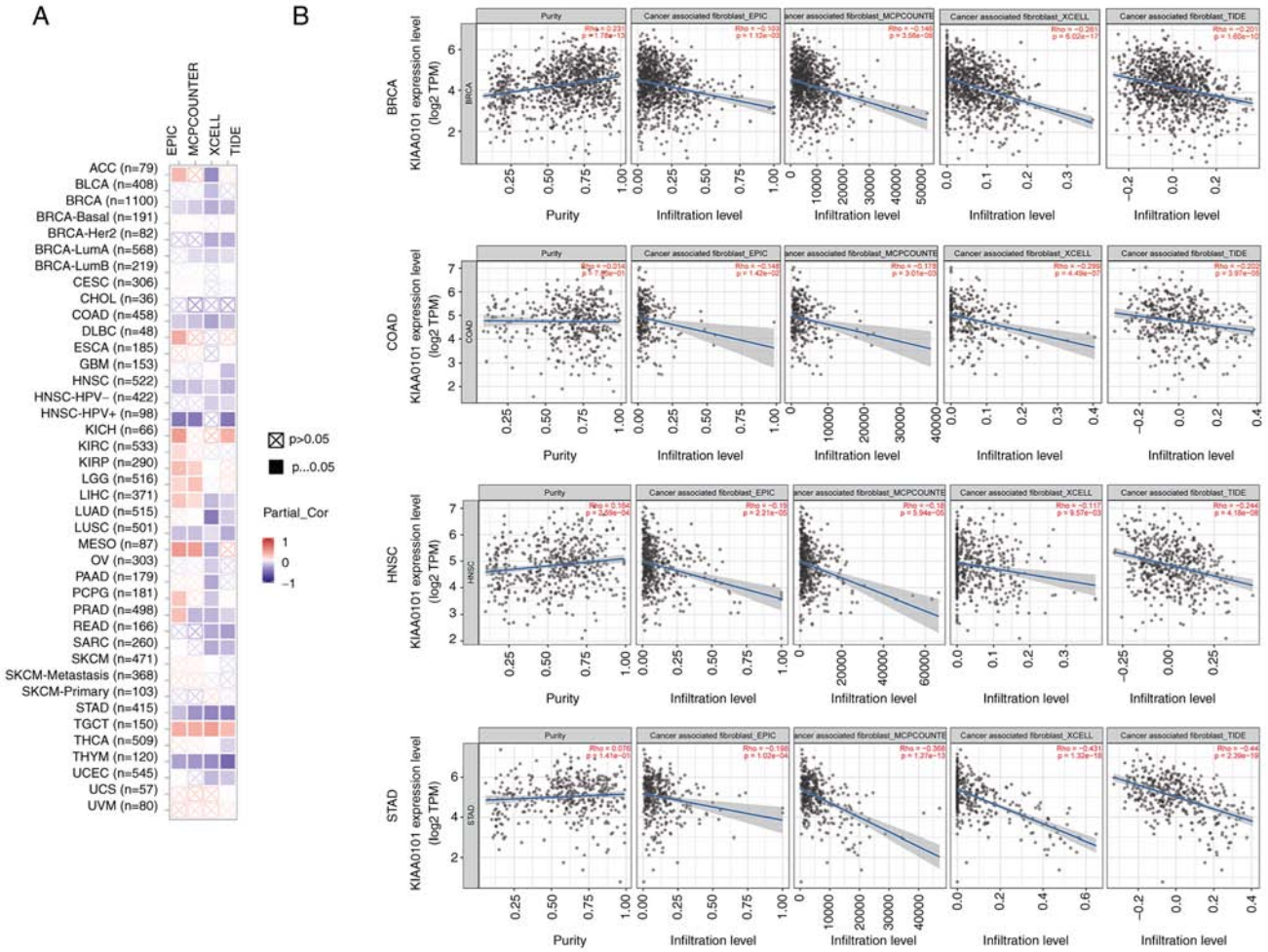

Figure 4. Correlation analysis between PCLAF expression and immune infiltration of cancer-associated fibroblasts. (A) EPIC, MCPCOUNTEER, XCELL, and TIDE algorithms were used to explore the correlation between the expression level of the *PCLAF* gene and the infiltration level of cancer-associated fibroblasts. (B) Correlation of PCLAF and infiltration level of cancer-associated fibroblasts across BRCA, COAD, HNSC, STAD. PCLAF, PCNA clamp associated factor, also known as KIAA0101.

that modifications and DNA copy variations were statistically independent of PCLAF expression (Fig. 3E). Therefore, the high expression of PCLAF in cancer may not be the result of genetic variation. Then we evaluated the epigenetic disorders of PCLAF in cancer. We found that eight high PCLAF-expressing tumors presented with decreased DNA methylation levels of PCLAF, including CHOL, CESC, GBM, PCPG, sarcoma (SARC), testicular germ cell tumors (TGCT), STAD, and UCEC (Fig. S4A). Since there is no available KICH DNA methylation dataset, we did not assess the overall DNA methylation level of KICH. On the contrary, we compared the DNA methylation level of KICH in different tumor stages and found no significant change in DNA methylation (Fig. S4B). In addition, the DNA methylation level of PCLAF in BRCA and COAD remained unchanged (Fig. S4C), suggesting that DNA methylation is not the only cause of abnormal expression of PCLAF.

Immune infiltration analysis data. With the bloom of tumor molecular biology and related disciplines, tumor-infiltrating immune cells, as an essential part of the tumor microenvironment, are closely related to occurrence, progression, and metastasis of malignant tumors (21,22). Components of the tumor microenvironment, containing endothelial cells, immune cells, and cancer-associated fibroblasts, play momentous roles in the formation of the extracellular matrix (ECM) and regulating disease progression (23,24). Herein, algorithms such as TIMER (27), CIBERSORT (37), CIBERSORT-abs, QUANTISEQ (38), XCELL (39), MCPCOUNTER (40), EPIC (41) are used to explore the potential relationship between different levels of immune cell infiltration in various tumor types of TCGA and *PCLAF* gene expression. A statistically positive association of PCLAF expression and the estimated infiltration value of cancer-associated fibroblasts was discovered for TCGA tumors. In contrast, PCLAF was negatively correlated in BRCA, COAD, HNSC, STAD, THYM and TGCT (Figs. 4A and B and S5A). The level of tumor infiltrating lymphocytes (TILs) can be performed as an independent predictor of sentinel lymph node status and cancer survival. Therefore, we also assessed the correlation between PCLAF expression and 28 TILs in the TISIDB database. Among the diseases in the TISIDB database, the expression of PCLAF was positively correlated with activated CD4 T cells (Act CD4) and type 2 T helper (Th2) cells (Fig. S5B and C). These data indicated that PCLAF may play a specific role in tumor immune infiltration. The TME (tumor microenvironment) plays a vital role in the occurrence and development of tumors, which may accelerate

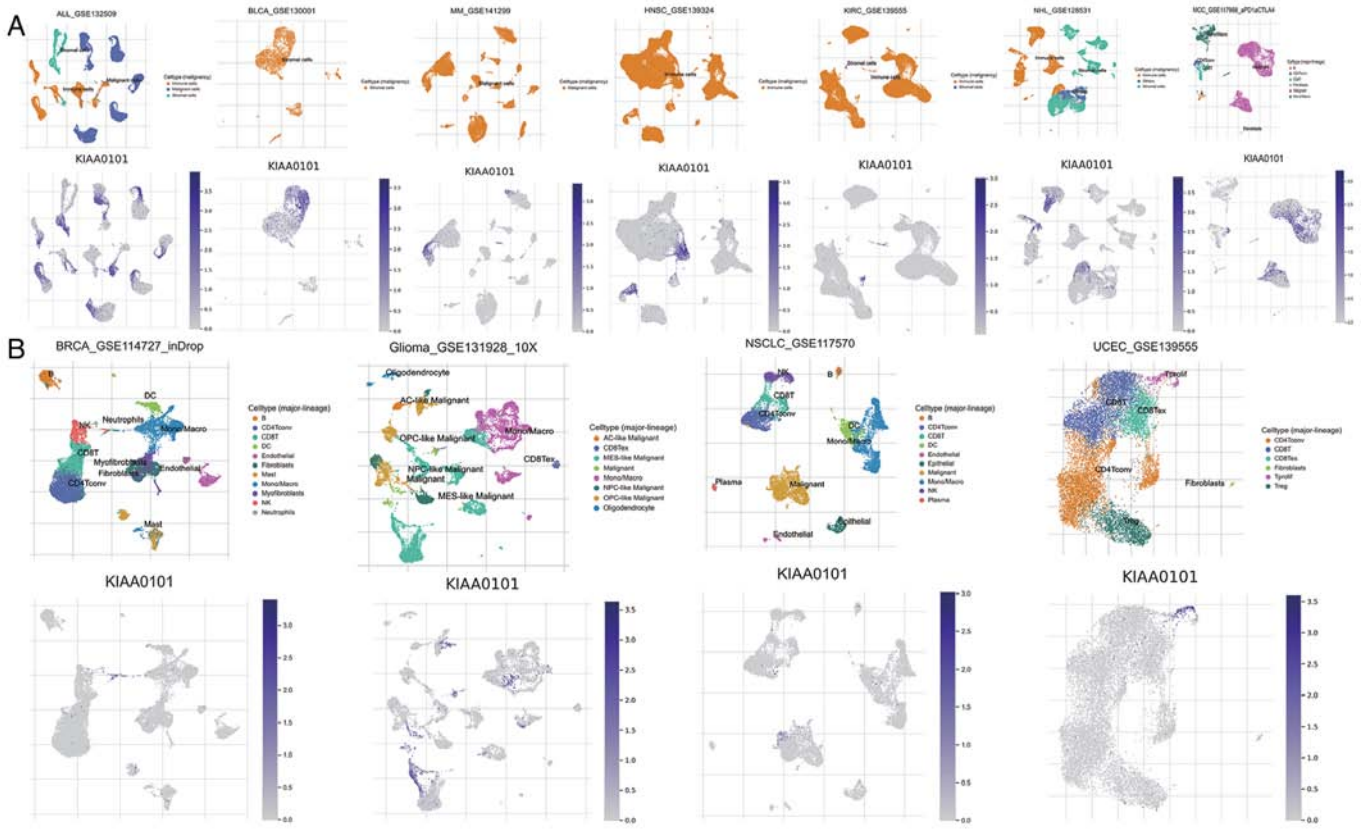

Figure 5. Correlation analysis between PCLAF expression and TME (tumor microenvironment). (A) PCLAF was expressed in immune cells, malignant cells, and stromal cells. (B) PCLAF expression was the highest in CD8 T cells, conventional CD4 T cells, exhausted CD8 T cells, monocytes and macrophages, and proliferating T cell fibroblasts in BRCA, Glioma, NSCLC, and UCEC. PCLAF, PCNA clamp associated factor, also known as KIAA0101.

the deterioration of tumors and affect the prognosis. We used the TISCH database to analyze the expression of PCLAF in TME-related cells. Among ALL, BLCA, MM, HNSC, KIRC, NHL, MCC, we found that PCLAF was expressed in immune cells, malignant cells, and stromal cells (Fig. 5A). In addition, PCLAF expression was the highest in CD8 T cells, conventional CD4 T cells, exhausted CD8 T cells, monocytes or macrophages, proliferating T cell fibroblasts in BRCA, Glioma, NSCLC, UCEC (Fig. 5B). These results demonstrated that PCLAF was closely related to TME in cancer.

Enrichment analysis of PCLAF-related partners. To further elucidate the underlying molecular mechanism of the *PCLAF* gene in tumors, we tried to screen out the targeted PCLAF binding protein and PCLAF expression-related genes by conducting a series of pathway enrichment analyses. In the STRING database, we obtained a total of 50 PCLAF-binding proteins, which are supported by evidence of co-expression. Fig. 6A shows the interaction network of these proteins. We used the GEPIA2 tool to combine all tumor expression data of TCGA to obtain the top 100 genes related to PCLAF expression. Then an intersection analysis was conducted among the PCLAF-binding and correlated genes by Venn (http://bioinformatics.psb.ugent.be/webtools/Venn/), and 36 common genes were obtained (Fig. 6B). The functions of 36 genes were predicted by analyzing GO and KEGG in Metascape.

We found that these genes are mainly enriched in the 'mitotic cell cycle process', 'cell division', 'Cell Cycle' and 'DNA replication' (Fig. 6C and D). As shown in Fig. 6E, the PCLAF expression level was positively correlated with that of BUB1 mitotic checkpoint serine/threonine kinase B (*BUB1B*) (R=0.60), cyclin B1 (*CCNB1*) (R=0.63), cell division cycle 45 (*CDC45*) (R=0.63), DLG associated protein 5 (*DLGAP5*) (R=0.60) and proliferating cell nuclear antigen (*PCNA*) (R=0.69) genes (all P<0.01). The heatmap data also shows that PCLAF is positively correlated with the above five genes (Fig. 6F). During mitosis, transcription factors (TFs) can maintain the ability to bind to target cells and nucleosome arrays. Due to the importance of PCLAF in cancer, we explored the TFs that regulate PCLAF. We obtained 20 TFs which regulate PCLAF in the CHEA database. These TFs were displayed as a bubble plot based on the correlation in 14 tumors (Fig. S6A and B). These TFs are mainly involved in the cell cycle, apoptosis regulation, DNA damage repair, and other pathways. In 75, 69 and 53% of selected cancer types, E2F1, FOXM1 and E2F7 may regulate PCLAF and activate cell cycle pathways (Fig. S6C and D). Previous research reported that FOXM1 could regulate PCLAF to predict poor prognosis in high-grade serous ovarian cancer patients (42).

PCLAF promotes the proliferation of liver cancer cells and inhibits apoptosis. In 2021, Kim *et al* reported that knockdown of *PCLAF* (KD) with shRNA inhibited the growth of lung tumor cells and the number of PCLAF-silenced lung cancer cells in G0/G1 phase was escalated (43). In 2018, Jin *et al* revealed that PCLAF accelerates ovarian cancer cell proliferation and PCLAF

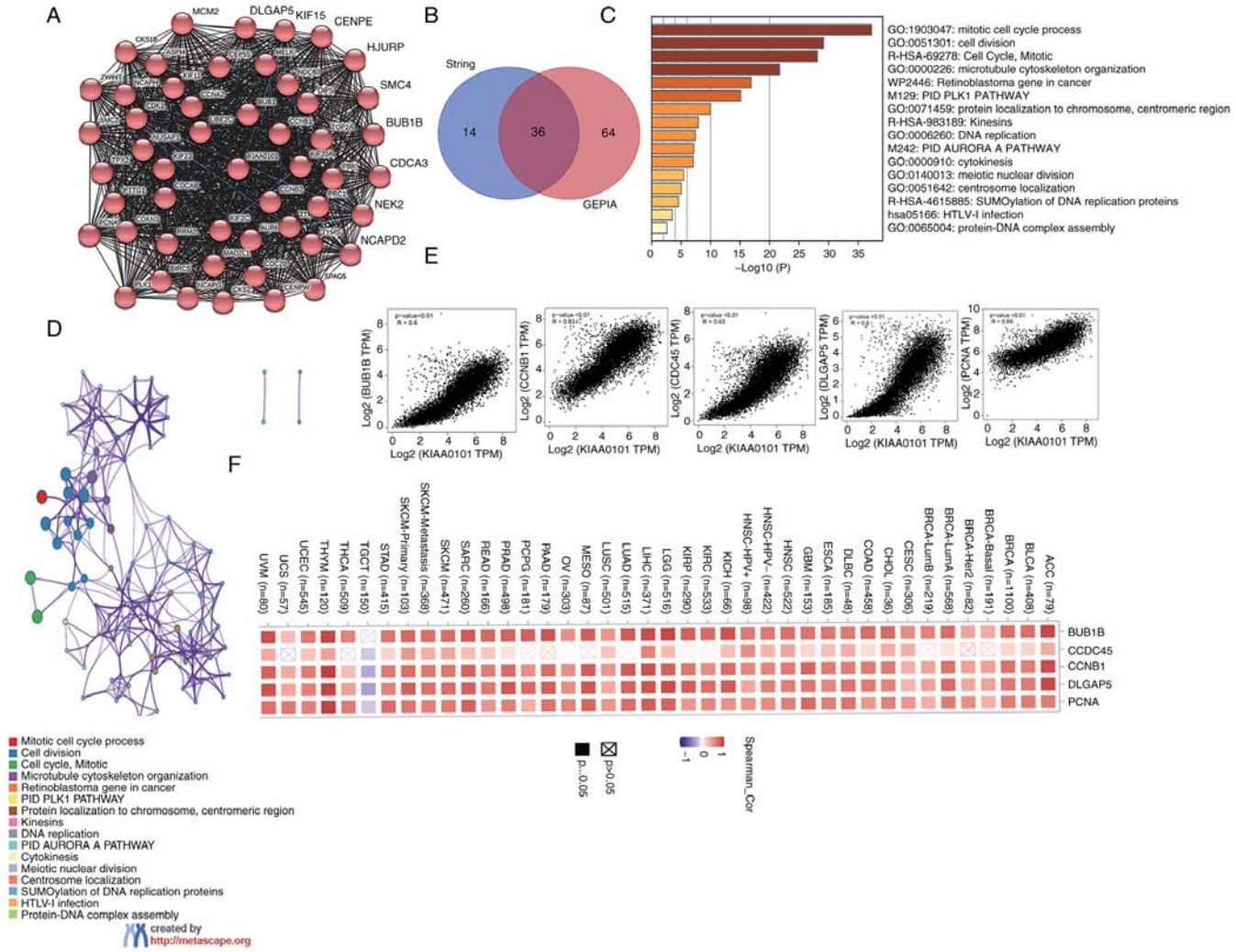

Figure 6. Enrichment analysis of PCLAF-related genes. (A) Interaction network of 50 PCLAF-binding proteins was experimentally determined by the STRING tool. (B) Intersection analysis of the PCLAF-binding and correlated genes was conducted. (C and D) Enrichment analysis of PCLAF and neighboring genes in Metascape. (E) The top 100 PCLAF-correlated genes in TCGA projects, including BUB1B, CCDC45, CCNB1, DLGAP5, and PCNA by the GEPIA2 approach. (F) Corresponding heatmap data in the exact cancer types are displayed. PCLAF, PCNA clamp associated factor, also known as KIAA0101.

knockdown reduced the percentage of cells in the S phase and augmented the percentage of cells in the G1 phase (42). In 2013, Jun *et al* revealed that downregulation of PCLAF inhibited the proliferation of pancreatic cancer Panc-1 cells and increased the proportion of cells in the G1 phase of the cell cycle (44). In addition, *PCLAF* (KD) can also abate the proliferation of gastric cancer, anaplastic thyroid carcinoma, glioma, breast cancer, colon cancer, adrenal cancer and nasopharyngeal carcinoma *in vitro* (13, 45-51). In order to better explore the function of PCLAF in cancer, we selected the liver cancer cell line (HepG2) for verification. We knocked down *PCLAF* by short hairpin RNA (shRNA), and performed the following experiments after verifying the interference efficiency (Fig. 7A). The HepG2 cell line was transfected with shPCLAF, and the CCK-8 assay was used to continuously monitor cell proliferation at 24, 48, 72, and 96 h after transfection. Interestingly, the cell viability was significantly decreased after the silencing of *PCLAF*, indicating that the reduction in *PCLAF* expression can inhibit the proliferation of liver cancer cells (Fig. 7B). At the same time, the cell cycle analysis results showed that the silencing of the expression of PCLAF in the HepG2 cell line significantly increased the percentage of cells in the G1 phase of the cell cycle (Fig. 7C). The results of the EdU experiment showed that after reducing the expression of *PCLAF*, the percentage of positive signals labeled with EdU was significantly reduced (Fig. 7D), indicating that the DNA replication activity of the cells was reduced and the cell proliferation ability was weakened. Apoptosis analysis showed that after the *PCLAF* gene was knocked down, the average percentage of apoptosis was significantly increased (Fig. 7E). Western blot analysis was further used to detect cell proliferation and apoptosis-related proteins. Attenuating the expression of PCLAF protein significantly downregulated cyclin A2, cyclin B1, cyclin D1, cyclin E2, CDK2, CDK6, and Bcl-2 and enhanced the expression of PARP, cleaved PARP, caspase 3, cleaved caspase 3, and Bax (Fig. 7F and G). In summary, the findings here further strengthen the conclusion that PCLAF has a broad-spectrum tumorigenic effect on various types of tumors.

Discussion

Increasing research has shown that the PCNA clamp associated factor (PCLAF) protein is involved in a series of cell

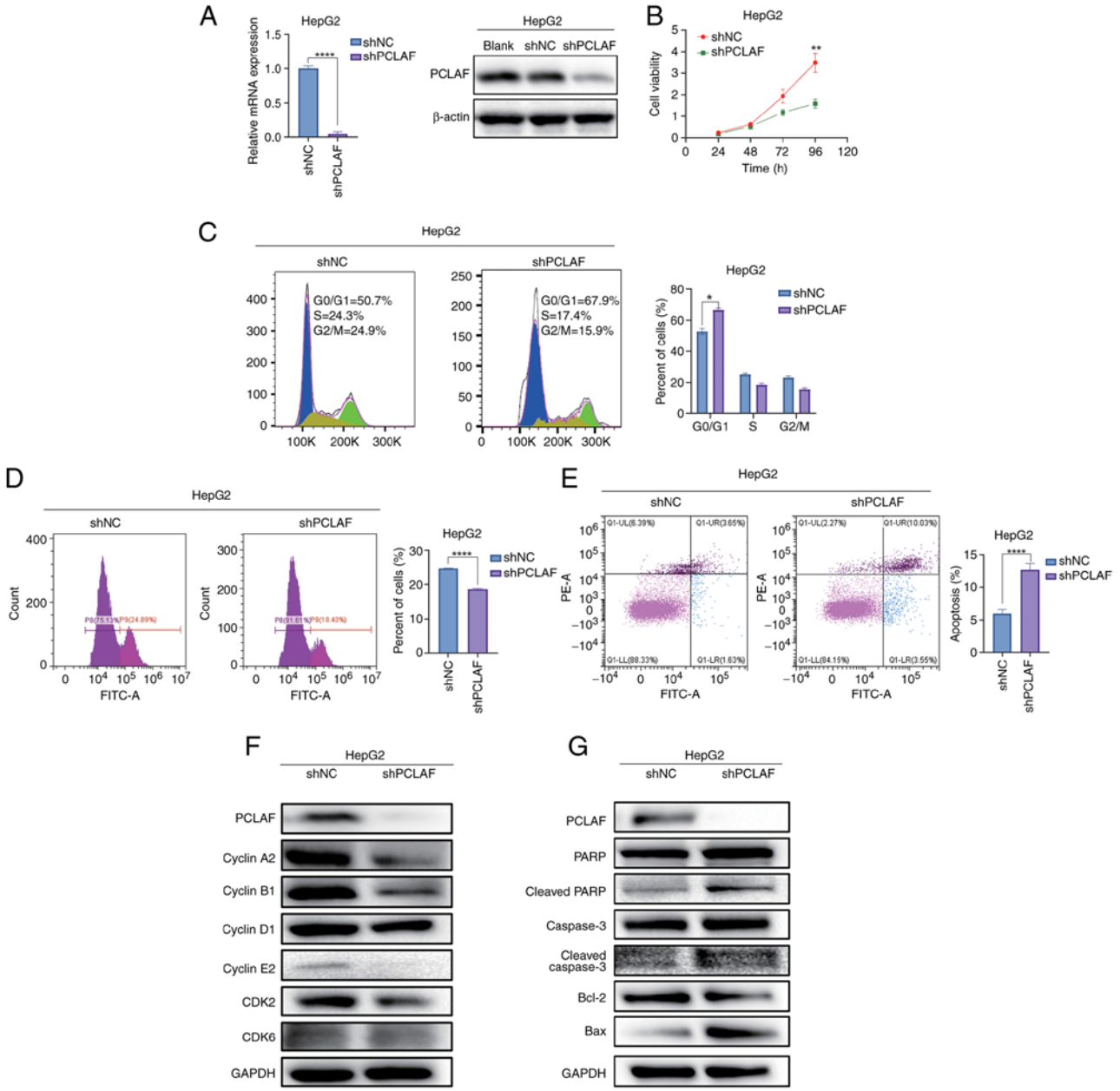

Figure 7. PCLAF promotes the proliferation of HepG2 cells and inhibit cell apoptosis. (A) qPCR and western blotting were used to verify the interference efficiency of shPCLAF in the HepG2 cell lines. (B) Growth curve was used to measure the effect of PCLAF on the proliferation of HepG2 cells. (C) Flow cytometry was used to analyze the cell cycle of HepG2 cells. (D) EdU labeling with flow cytometry was used to detect the proliferation function of PCLAF *in vitro*. (E) Flow cytometry was used to detect cell apoptosis after *PCLAF* knockdown. (F and G) Western blot analysis was used to detect cell cycle-and apoptosis-related markers in HepG2 cells (*P<0.05, **P<0.01, and ****P<0.0001; bar graphs represent the mean ± SEM). PCLAF, PCNA clamp associated factor, also known as KIAA0101.

biological events, such as cell cycle regulation, DNA replication, DNA repair, and cell survival. More and more studies have confirmed the functional interaction between PCLAF and tumors (8,10,11,52,53). Numerous studies have performed immunohistochemical analysis of various tumor tissues, which strongly suggest that PCLAF is highly expressed in a variety of tumor tissues and may serve as a pan-cancer prognostic biomar ker (13,42,43,46-50,54,55). Yet, the pathogenesis of PCLAF in different tumors is still unclear, and further research is needed. After a comprehensive literature search, we did not find any publications concerning PCLAF pan-cancer analysis. Our research illustrates that computational biology can discover the molecular biological mechanisms by which PCLAF affects tumor progression. In the present study, PCLAF was found to play a prognostic role in the pan-cancer and tumor microenvironment, which provides clues to understand the prognosis and immune effects of PCLAF in different tumors.

The present study used the TCGA data of ONCOMINE, GEPIA, and TIMER to explore the expression levels of PCLAF in different tumors and to visualized its prognosis in pan-cancer. In ONCOMINE, we found that the expression level of PCLAF was only low in leukemia, and other tumors showed high expression status. The TCGA data analysis in TIMER showed that PCLAF in BLCA, BRCA, CESC, CHOL, COAD, ESCA, GBM, HNSC, -HPV+ Tumor, KICH, KIRC, KIRP, LIHC, LUAD, LUSC, PCPG, PRAD, READ, SKCM, STAD, THCA, and UCEC is higher than

that noted in normal adjacent tissues. In addition, we found that the expression of PCLAF increased with the clinical stage from stage I to stage IV, including ACC, KICH, KIRC, KIRP, LIHC, LUAD, LUSC, and PAAD. We used the GEPIA2 tool to analyze the relationship between *PCLAF* gene expression and the overall survival (OS) and disease-free survival (DFS) of different tumors in TCGA. We found that high expression of PCLAF was associated with poor OS in ACC, KIRC, KIRP, LGG, LIHC, LUAD, MESO, and PAAD. DFS analysis data showed that in ACC, KIRP, LGG, LIHC, LUAD, MESO, PRAD, and UVM tumors, patients with high PCLAF expression had a worse prognosis. In summary, these findings strongly indicate that PCLAF can be used as a biomarker for pan-cancer prognosis.

It is generally believed that cancer is caused by genetic mutations, which biologically enhance the resistance of cancer cells to surrounding normal cells (56-58). At present, advances in systems biology methods provide us with a large amount of data to identify molecular alterations and explore the heterogeneity of cancer cells (59-61). We explored the mutation pattern and amplification frequency of PCLAF in different tumors by using the CbioPortal tool. We found that the most common DNA change in the TCGA pan-cancer group was amplification. Then we analyzed the correlation between genetic diseases and PCLAF expression and found that mutations have nothing to do with RNA expression status. In addition, we found that mutations and DNA copy variations were also independent of PCLAF expression. Therefore, genetic variation may not be the factor that causes the high expression of PCLAF in tumors. Then we assessed the epigenetic disorders of PCLAF in cancer and found that aberrant DNA methylation may be the cause of abnormal expression of PCLAF in tumors, but it is not the only cause.

Another important aspect of this study is that the expression of PCLAF is associated with diverse levels of immune infiltration in cancer. In TGCT tumors, we observed a statistical positive correlation between the estimated infiltration value of cancer-associated fibroblasts (CAFs) and PCLAF expression. At the same time, it was statistically negatively correlated in BRCA, COAD, HNSC, STAD, THYM and TGCT. In the diseases in the TISIDB database, the expression of PCLAF was found to be positively correlated with activated CD4 T cells (Act CD4) and type 2 T helper (Th2) cells, suggesting that PCLAF may play a specific role in tumor immune infiltration. Mounting evidence has demonstrated that the tumor microenvironment (TME) plays a predominant role in the occurrence and development of tumors, which may accelerate the deterioration of tumors (62,63). Among the TISIDB database, in BRCA, Glioma, NSCLC, and UCEC, PCLAF was found to be highly expressed in CD8 T cells, regular CD4 T cells, CD8-poor T cells, monocytes or macrophages, and proliferating T cell fibroblasts, which suggests that PCLAF is closely related to tumor TME.

In addition, the information on PCLAF-binding components and PCLAF expression-related genes from all tumors was integrated. A series of identified biological terms were markedly enriched, which characterized processes related to 'mitotic cell cycle process', 'cell division', 'cell cycle', and 'DNA replication'. Decreasing the expression of PCLAF can inhibit the proliferation of undifferentiated thyroid cancer and cervical cancer cell lines, DNA synthesis and cell viability of pancreatic cancer cell lines, leading to an increase in the number of G0/G1 cells in adrenocortical cancer cell lines and cervical cancer cell lines (6,43,46). These findings indicate that PCLAF may cause cancer cell proliferation by promoting cell cycle progression. We obtained 20 transcription factors (TFs) regulating PCLAF, mainly involved in the cell cycle, apoptosis regulation, DNA damage repair, and other pathways. The above results indicate that PCLAF participates in carcinogenesis under the regulation of these TFs. Most importantly, our analysis of liver cancer cell lines indicated that PCLAF enhanced the proliferation of liver cancer cells and inhibited cell apoptosis *in vitro*, but the mechanism by which PCLAF promotes tumor cell proliferation will require further exploration.

In summary, our first pan-cancer analysis of PCLAF demonstrated that PCLAF expression is statistically correlated with clinical prognosis, DNA methylation, and immune cell infiltration, which aids in understanding the role of PCLAF in tumorigenesis from the perspective of clinical tumor samples.

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Availability of data and materials

All data included in this study are available by contacting the corresponding authors.

Authors' contributions

XL conceived and designed the study and drafted the manuscript. YC and CC explained and prepared the data. XL, YG, and YW collected and analyzed the data. KC and ZW revised the manuscript and finally approved the version to be published. All authors read, validated the data generated and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no potential competing interests.

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