Polo-like kinase 1 plays an oncogenic role in pan-cancer based on bioinformatics and biological assays

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Research Article

Keywords: pan-cancer, glioma, PLK1, prognosis, alteration, methylation, immune infiltration

Posted Date: March 22nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1418158/v1

Abstract

Polo-like kinases (PLKs) are crucial regulatory factors of the cell cycle process and their dysregulations often cause various diseases including tumorigenesis. Although the PLKs, including PLK1, have been extensively studied and reported, few pan-cancer analyses are attainable. The potential oncogenic roles of PLK1 were thus explored among different cancers according to various online websites and datasets. PLK1 is always overexpressed in many cancers, and there is a significant relationship between its expression and the prognosis of cancer patients. In addition, the enrichment analysis suggested that PLK1 might related to “cell cycle”, “DNA replication”, “mismatch repair”, and “immune response” in pan-cancer, especially in glioma. Therefore, the analyses about genetic alterations and immune in pan-cancer were conducted. Furthermore, flow cytometry, CCK8 and EdU assays can further verify the above results of enrichment analysis. Moreover, DNA methylation and ceRNA network were analyzed to explore the potential mechanisms of aberrant expression of PLK1. In conclusion, our research about the oncogenic roles of PLK1 in pan-cancer further supplemented the oncogenic mechanisms of PLK1 and developed new potential therapeutic directions.

Introduction

One of the most striking features of cancer cells is that aberrant cell cycle leads to their uncontrolled cell proliferation, which is usually caused by aberrant expression of the cell cycle-related genes[1]. In recent years, more and more cell cycle-related genes were emerging as the candidate biomarkers for early diagnosis and potential therapeutic targets in cancers[2, 3].

Polo-like kinase (PLK) family has aroused our research interests because of its close relationship with cell cycle[4–6]. PLKs belong to the serine/threonine kinase family that play differentiated and critical roles as key cell cycle regulators in tumor genesis and development[7, 8]. PLKs are widely distributed in eukaryotic cells and the human PLKs family consists of five members, including PLK1, PLK2, PLK3, PLK4, and PLK5[9].

Polo-like kinase1 (PLK1) is a highly conservative serine/threonine kinase widely found in eukaryotic cells and plays crucial roles in the cell cycle process[10]. PLK1 is characterized by C-terminal serine domain[7], which can regulate N-terminal serine/serine kinase domain, mediates protein interaction and intracellular localization. PLK1 is also responsible for a wide range of cellular functions. It plays an important role in centriole maturation[11–13], Golgi disintegration[14], spindle assembling and function[15, 16], kinetochore function[17, 18], centromere assembling[19] and cytokinesis[20]. It also facilitates DNA replication[21], mitotic entry[22], separation of sister chromatid[23], chromosome condensation[24] and APC/C activity[25]. It has been reported that PLK1 is frequently over-expressed in numerous cancers (such as esophageal cancer, colon cancer, breast cancer, non-small cell lung cancer, endometrial cancer, etc.), and facilitates the occurrence and progress of these cancers acting as an oncogene[26]. Although there has been a mass of researches on the roles of PLK1 in tumorigenesis and development, few

analyses of PLK1 in pan-cancer have been conducted. To remedy this deficiency, we conducted an analysis of PLK1 in pan-cancer, especially in glioma.

In this present study, we have comprehensively explored the PLK1 expression and its relationship with the prognosis of tumor patients in pan-cancer, especially in glioma across some datasets. And 100 glioma samples were obtained to further validate the correlation between PLK1 mRNA expression and glioma grades or prognosis. Besides, the hypothesis that PLK1 is closely related to genetic alterations, immune, and cell cycle in tumors was also supported by KEGG and GO enrichment analysis of its related genes in pan-cancer, especially in glioma. And it was confirmed that PLK1 might be related to the occurrence and progression of glioma by bioinformatic analysis. Some experiments such as flow cytometry, CCK8 and EdU also confirmed that aberrant expression of PLK1 lead to the occurrence and progression of glioma by regulating the cell cycle. In addition, we also explored the potential mechanisms of aberrant expression of PLK1 by analyzing PLK1 methylation and ceRNA network.

Materials And Methods

2.1 Experimental methods

2.1.1 Cell culture and transfection

Human astrocyte and human GBM (glioblastoma multiforme) cell lines (U87, U251, LN229, A172 and B19) were obtained from ATCC (the American Type Culture Collection, Manassas, VA, USA). The human astrocyte was grown in Astrocyte Medium (AM) (ScienCell, USA) medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA) and human GBM cell lines (U87, U251, LN229, A172 and B19) were grown in DMEM medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA). Cell lines were tested using the ATCC cell line authentication service and routinely tested for Mycoplasma. All cells have been growing at 37 °C in a humidified atmosphere (95 % humidity) with 5 % CO_2[27].

In knockdown experiment, GBM cells (U87 and LN229) were treated with PLK1-siRNA (GenePharma, Shanghai, China) by using Lipofectamine RNAimax (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. PLK1 siRNA 1#: 5'-TCATTAAGCAGCTC-3'; PLK1 siRNA 2#: 5'-CATTAAGCAGCTCG-3'.

2.1.2 RNA isolation and quantitative real-time PCR

The expression of mRNA in the cancer cell lines was detected by qRT-PCR. The total RNA of the cells was extracted using the TRIzol (Thermo Fisher Scientific, USA). The mRNA (1 μg) was reverse transcribed into cDNA by the reverse transcription kit (Promega, USA). The expression status of mRNA was measured on ABI QuantStudio 3 using GoTaq® qPCR Master Mix (Promega, USA). GAPDH was selected as the loading control for mRNA expression analyses. cDNA product (2 μl) was used as template in a 20 μl PCR system containing 10 μl of GoTaq® qPCR Master Mix and 2 μl of each primer. All reactions were performed in

duplicate. Amplification protocols were as follows: 95°C for 10 min; 44 cycles of 95°C/10 s, 58°C/10 s, and 60°C/10 s. The primer sequences were as following: PLK1 Forward: 5'- AAGAGATCCCGGAGGTCCT-3', Reverse: 5'-TCATTCAGGAAAAGGTTGCC-3'. GAPDH Forward: 5 -GGAGCGAGATCCCTCCAAAAT-3 Reverse: 5 -GGCTGT TGTCATACTTCTCATGG-3 . Data were analyzed using the relative standard curve method and normalized to GAPDH[28, 29].

2.1.3 CCK8 assay

CCK8 assay (Dojindo, Japan) was used to detect cell proliferation. Each group of cells was seeded into 96-well plates. Six replicate wells were set for each group. The number of cells in each well was about 2×10^4. After cultured for 0days, 1 day, 2 days, 3 days, 4 days and 5 days, 10 μL of reagent CCK-8 (ApexBio, USA) were added, and the cells were incubated at 37℃ with 5% CO_2 for 2 hours. The optical density (OD) values were obtained via a microplate reader at 450 nm wavelength using Varioskan TM LUX microplate reader (Thermo Fisher Scientific, USA). The cell viability was calculated as: [OD (Test) - OD (Blank)] / [OD (Control) - OD (Blank)] × 100%.

2.1.4 EdU assay

EdU assay was used to examine cell proliferation. Glioma cell lines in the logarithmic growth phase were seeded into 96-well plates at a density of 2×10^3- 4×10^4. After 24 h, the adherent cells to the wells were transfected. Five parallel wells were set up for each group. Cells in each well after transfection for 48 h were cultured with 100 μL EdU medium (RIBOBIO, China) for 2 h and fixed with 100 μL of cell fixation solution (PBS containing 4% polyformaldehyde) for 30 min at room temperature. Subsequently, the cells were incubated with 2 mg/mL glycine (Solarbio, China) for 5 min, rinsed with 100 μL of PBS containing 0.5% TritonX-100 (RIBOBIO, China) for 10 min, and stained using 1 × Apollo staining reaction solution (RIBOBIO, China) for 30 min in conditions devoid of light. Next, the cells were reacted with 100 μL of the 1 × Hoechst 33342 reaction solution (RIBOBIO, China) for 30 min and sealed with 100 μL of the anti-fluorescence quenching agent. Six to ten fields of view were randomly selected for each well and photographed under a fluorescence microscope[9].

2.1.5 Flow cytometry

Cell cycle arrest was analyzed by flow cytometry. The collected cells were washed with precooled 1×PBS for 3 times, and the supernatant was discarded after cell precipitation by centrifugation. After resuspend the cells with 0.5 mL 1×PBS, 3.5 mL 70% ethanol precooled was added quickly, beaten evenly, and stored overnight at 4℃. The supernatant of cells fixed by centrifugal ethanol was discarded, and then washed with 1×PBS for 3 times to remove the residual ethanol. The cells were resuspended with 1 mL Pi /Triton X-100 staining solution containing 0.2 mg RNase A (20 μg Pi /0.1% Triton X-100) and then stained at 37℃ for 15 min. Finally, the cell cycle was measured by flow cytometry (Beckman Coulter, USA)[30].

2.1.6 RNA-seq

RNA-Seq data (Illumina HiSeq X Ten, Novogene) and corresponding pathological and clinical data of external 100 glioma samples were obtained to further validate the correlation between PLK1 mRNA expression and glioma grades or prognosis. All human glioma samples were taken from patients undergoing surgery at the First Affiliated Hospital of Zhengzhou University (Supplementary Table 1). Tissue samples were graded by neuropathologists according to World Health Organization (WHO) standards and stored in liquid nitrogen. Glioma specimens were divided into LGG (50 cases) and GBM (50 cases). The human glioma samples were approved by the institutional review boards of the First Affiliated Hospital of Zhengzhou University and obtained written informed consent from all patients.

2.2 Bioinformatic analyses

2.2.1 HPA: The Human Protein Atlas

The Human Protein Atlas (HPA) (https://www.proteinatlas.org/) is a Swedish project launched in 2003 to map all human protein in cells, tissues and organs by integrating various omics technologies, including antibody-based imaging, mass spectrum-based protein omics, transcriptomics and systems biology[31-33].

The HPA online website was used to analyze PLK1 in “Tissue Atlas”, “Single Cell Type Atlas” and “Cell In Atlas” module. The expression data of PLK1 mRNA in different human normal tissues and tumor/non-tumor cells were obtained. The row data source was TMM normalized. Normalized eXpression (NX), the resulting transcript expression values, were calculated for each gene in every sample. Online website (https://www.proteinatlas.org/about/assays+annotation) showed the detailed information.

2.2.2 The Oncomine database

The expression levels of PLK1 gene in different tumor types were investigated in Oncomine (https://www.oncomine.org/resource/login.html). The parameters were shown below: P-value<0.001, FC (fold change)>1.5 and gene rank is all[34, 35].

2.2.3 The online platform: SangerBox

We obtained a simulation map of the subcellular localization of the PLK1 protein using the SangerBox online tool (http://SangerBox.com/Tool). The COX_OS (overall survival) and COX_DFS (disease free survival) analysis data of PLK1 for different tumors was analyzed on SangerBox portal. In addition, the SangerBox online tool can also perform PLK1-related s KEGG and GO enrichment analysis.

2.2.4 GEPIA2: Gene Expression Profiling Interactive Analysis 2.0

The online website: Gene Expression Profiling Interactive Analysis 2.0 (GEPIA2) (http://gepia2.cancer-pku.cn/#index) is an interactive web that includes 9,736 tumors and 8,587 normal samples from TCGA and the GTEx databases, which analyses the RNA sequencing expression[36, 37]. In this present study, GEPIA2 was also used to conduct survival curves, including overall and disease-free survival in 33

different cancer types. The correlation analysis of gene expression was conducted using the given TCGA expression dataset. The correlation coefficient was determined by Spearman's statistical method.

2.2.5 Chinese Glioma Genome Atlas (CGGA) and The Cancer Genome Atlas (TCGA) datasets

The RNA-seq data and clinical information of CGGA-325 (http://www.cgga.org.cn/download.jsp) are shown in Supplementary Table 4; The RNA-seq data and clinical information of CGGA-693 (http://www.cgga.org.cn/download.jsp) are shown in Supplementary Table 5; The RNA-seq data and clinical information of TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) are shown in Supplementary Table 6;

2.2.6 The STRING database

We entered the STRING website (https://string-db.org/) using the query of a single protein name (“PLK1”) and organism (“Homo sapiens”). Next, we optioned the threshold as following: minimum required interaction score [“Low confidence (0.150)”], meaning of network edges (“evidence”), max number of interactors to show (“no more than 50 interactors” in 1st shell) and active interaction sources (“experiments”). At last, the PLK1-binding proteins and related PPI network that had been experimentally confirmed were obtained[38, 39].

2.2.7 The GEO databases

GSE67102 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67102) and GSE46856 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46856) databases were used to analyze the biological functions of PLK1 related genes. The differentially expressed genes were obtained after treating with inhibitors of PLK1 in GSE67102 and GSE46856 databases. Then KEGG and GO-BP enrichment analyses of PLK1 related genes was analyzed and mapped.

2.2.8 The cBioportal website

After logging into the cBioPortal website (https://www.cbioportal.org/), we chose the “TCGA Pan Cancer Atlas Studies” in the “Quick select” section and entered “PLK1” to query of the gene alteration characteristics of PLK1. We obtained the results of the alteration frequency, mutation type and copy number alteration (CNA) among all TCGA tumors in the “Cancer Types Summary” module. The information on the mutations site of PLK1 can be displayed in the protein structure diagram or 3D structure through the "Mutilations" module[40, 41].

2.2.9 The web portal TISIDB

TISIDB (http://cis.hku.hk/TISIDB/index.php) is an online website that integrates tumor and immune system interactions across multiple heterogeneous data types[42]. We got the relationship between the abundance of tumor-infiltrating lymphocytes (TILs) and the PLK1 mRNA levels in this study.

2.2.10 Genomic Identification of Significant Targets in Cancer (GISTIC) 2.0

Databases of somatic mutations and somatic copy number alternations (CNAs) were obtained from TCGA datasets. CNAs correlated with PLK1 mRNA levels, and the threshold copy number at alteration peaks were analyzed by GISTIC 2.0 (https://cloud.genepattern.org/). The patients were divided into the first 25% PLK1-low (n=170) and the last 25% PLK1-high (n=170) groups according to the expression value of PLK1. The maftools package was also used in R software (https://www.r-project.org/) to download and visualize the somatic mutations.

2.2.11 Immune Cell Abundance Identifier (ImmuCellAI)

ImmuCellAI (http://bioinfo.life.hust.edu.cn/ImmuCellAI#!/) portals were used to visualize the relationship between PLK1mRNA levels and tumor-related immune cells in GBM via CGGA and TCGA datasets.

2.2.12 The UALCAN database

The UALCAN portal (http://ualcan.path.uab.edu/analysis-prot.html), an interactive web resource, is usually used to analyze cancer Omics data[43]. In present study, we used it to compare the differential expressions of PLK1 protein in a specific tumor and its corresponding normal tissue and the methylation levels of the PLK1 promoter region between some primary tumors and normal tissues

2.2.13 Statistical Analysis

Through the online databases mentioned above, the statistical analysis was automatically computed in this study. These results were considered as statistically significant at *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

2.2.14 Ethics approval and consent to participate

Our research was approved by the Human Scientific Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1975 Helsinki declaration and its later amendments or comparable ethical standards. An informed consent was obtained from all individual participants included in the study.

Results

3.1 The expression pattern of PLK1 mRNA in pan-cancer

The flowchart of this study is shown in Supplementary Figure 1. The Normalized eXpression (NX) levels of PLK1 were analyzed in various tumor tissues and their corresponding adjacent normal tissues as well as various tumor cells and the corresponding non-tumor cells in the Human Protein Atlas (HPA) database. PLK1 mRNA expression levels were higher in the normal human thymus, testis, and tonsil (NX>20; Figure 1A). In most other normal human tissues, PLK1 mRNA expression levels were detectable but low (NX<20) (Figure 1A). PLK1 mRNA expression levels were higher in the early spermatids, extravillous trophoblasts

and erythroid cells (NX>20; Figure 1B). In most other normal human cells, PLK1 mRNA expression levels were detectable but low (NX<20) (Figure 1B). In human tumor cell lines, the expression level of PLK1 mRNA was the most abundant in human hepatocellular carcinomas cell lines (Hep G2), followed by human leukemia cell lines (HAP1) (Figure 1C). Moreover, in order to learn the differences of PLK1 mRNA expression in cancer and normal tissues, we analyzed the expression levels of PLK1 mRNA in different tumor tissues and normal tissues through the Oncomine website. The results suggested that the expression levels of PLK1 mRNA were higher in bladder, brain and CNS (Central Nervous System), colorectal, gastric, breast, esophageal, cervical, head and neck, ovarian, lung, liver, pancreatic cancer and lymphoma, sarcoma, leukemia compared to the normal tissues (Figure 1D). The integrated conditions of PLK1 expression in various cancers were collected in Supplementary Table 2. To further learn the PLK1 mRNA expression condition in different cancers, we tested the PLK1 mRNA expression across the RNA-seq data of a variety of malignancies in TCGA. There displayed the mRNA expression levels of PLK1 in all TCGA tumors (Figure 1E). There is a significant difference of PLK1 mRNA expression levels among TCGA tumors (Figure 1E). Moreover, we further analyzed the differential expressions of PLK1 mRNA between tumor tissues and normal tissues using the TCGA and GTEx databases with SangerBox. The expression of PLK1 mRNA was statistically higher in 24 cancers: adrenocortical carcinoma(ACC), bladder rothelial 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 cancer (HNSC), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), brain lower grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TGCT), uterine corpus endometrial carcinoma (UCEC), and uterine carcinosarcoma (UCS). Whereas, the PLK1 mRNA expressions were lower in acute myeloid leukemia (LAML) and thyroid carcinoma (THCA) and (Figure 1F).

Next, we analyzed PLK1 mRNA expression levels in different WHO grades and 2021 WHO classifications of gliomas. The gliomas are classified into the following three types according to the fifth edition of the World Health Organization (WHO) classification of tumors of the Central Nervous System (CNS) (WHO CNS5): IDH-mutant and 1p/19q-codeleted (mut+codel) oligodendroglioma, IDH-mutant and 1p/19q-noncodeleted (mut+non-codel) astrocytoma, and IDH-wildtype (IDH-wild) glioblastoma. The results showed that PLK1 expression levels were positively associated with glioma grades CGGA and TCGA databases (Supplementary Figure 2A). And the receiver operating characteristic (ROC) curve verified that PLK1 could be an effective factor for predicting WHO grades of glioma (Supplementary Figure 2B). Moreover, the expression levels of PLK1 mRNA correlated with the WHO CNS5 types of gliomas (Supplementary Figure 2C). Isocitrate dehydrogenase (IDH) mutations and chromosomal 1p/19q codeletions are associated with better survival outcomes of glioma patients. Furthermore, promoter methylation status of the O6-methylguanine DNA methyltransferase (MGMT) is a prognostic indicator of the clinical response to treatment of glioblastoma patients with temozolomide (TMZ). Then, we explored

the relationship between PLK1 mRNA expression and the status of IDH gene mutations, 1p/19q codeletion, and MGMT promoter methylation. Analysis of the CGGA-325, CGGA-693 datasets and TCGA datasets showed that PLK1 mRNA levels in the glioma patients with wild-type IDH and chromosomal 1p/19q non-codeletion were significantly higher (Supplementary Figure 2D-E), however, there is no significantly difference in the glioma patients with MGMT promoter methylation (Supplementary Figure 2F).

In conclusion, the above results suggested that PLK1 mRNA levels were upregulated in several tumors. Furthermore, PLK1 expression levels correlated with the grades, the WHO CNS5 types, and clinical features of gliomas.

3.2 The expression pattern of PLK1 protein in pan-cancer

In this part, our aim was to explore the oncogenic roles of the human PLK1 protein. We investigated the expression characteristics of the PLK1 protein in 41 different normal tissues and various cancers using the HPA database, respectively. The analysis results appeared that the expression of PLK1 protein in human normal tissues was highest in testis, while the expression in other tissues was low or moderate (Figure 2A). Whereas, PLK1 protein in human cancer tissues was highest in thyroid cancer, while the expression in glioma tissues was moderate (Figure 2B). The conserved analysis of PLK1 protein among different species showed that the amino acid sequence and domain of PLK1 protein is conserved among different species (Figure 2C-D). The phylogenetic tree figure presented the evolutionary relationship of the PLK1 proteins among various species (Figure 2E). The CPTAC database of UALCAN online tool was used to explore the differential expressions of PLK1 proteins in tumor and normal tissues. The analysis results appeared that the PLK1 protein expression levels in uterine corpus endometrial carcinoma (UCEC), colon cancer, and lung adenocarcinoma were overexpressed than that in normal tissues (Figure 2F).

These results suggested that the domain of PLK1 protein is conserved between different species, and PLK1 protein may play a carcinogenic role in some tumors, such as uterine corpus endometrial carcinoma, colon cancer and lung adenocarcinoma.

3.3 PLK1 expression is associated with the prognosis in pan-cancer including gliomas

First, we analyzed the correlation between PLK1 mRNA levels and prognosis in pan-cancer by GEPIA2 with TCGA database and plotted survival curves for overall survival (OS) and disease-free survival (DFS) respectively. The results showed that higher PLK1 mRNA levels were statistically related to poorer OS and DFS in pan-cancer (Figure 3A-B). Then we further analyzed the correlation between the expression levels of PLK1 mRNA and prognosis in specific tumor types. The analysis appeared that higher PLK1 mRNA expression levels were statistically related to the poorer OS of adrenocortical carcinoma (ACC), breast invasive carcinoma (BRCA), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), brain lower grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), Mesothelioma (MESO), pancreatic adenocarcinoma (PAAD), skin cutaneous melanoma (SKCM) (Figure 3C), and poorer DFS of adrenocortical carcinoma (ACC), breast

invasive carcinoma (BRCA), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), brain lower grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), Mesothelioma (MESO), pancreatic adenocarcinoma (PAAD), prostate adenocarcinoma (PRAD), Sarcoma (SARC), skin cutaneous melanoma (SKCM), thyroid carcinoma (THCA), uveal melanoma (UVM) (Figure 3D).Cox regression analysis of the SangerBox database showed that PLK1 mRNA levels were associated with OS and DFS of patients with multiple cancers. The results showed that the high mRNA levels of PLK1 were associated with shorter OS in breast invasive carcinoma (BRCA), Sarcoma (SARC), skin cutaneous melanoma (SKCM), head and neck squamous cell carcinoma (HNSC), skin cutaneous melanoma-metastasis (SKCM-M), lung adenocarcinoma (LUAD), liver hepatocellular carcinoma (LIHC), pancreatic adenocarcinoma (PAAD), kidney renal clear cell carcinoma (KIRC), brain lower grade glioma (LGG), Mesothelioma (MESO), Pan-kidney cohort, (KIPAN), glioma (GBM+LGG), cholangiocarcinoma (CHOL), kidney chromophobe (KICH), adrenocortical carcinoma (ACC), and kidney renal papillary cell carcinoma (KIRP) and shorter DFS in Sarcoma (SARC), skin cutaneous melanoma (SKCM), skin cutaneous melanoma-metastasis (SKCM-M), breast invasive carcinoma (BRCA), lung adenocarcinoma (LUAD), liver hepatocellular carcinoma (LIHC), pancreatic adenocarcinoma (PAAD), uveal melanoma (UVM), brain lower grade glioma (LGG), cholangiocarcinoma (CHOL), kidney renal clear cell carcinoma (KIRC), glioma (GBM+LGG), Mesothelioma (MESO), adrenocortical carcinoma (ACC), kidney chromophobe (KICH), prostate adenocarcinoma (PRAD), and kidney renal papillary cell carcinoma (KIRP) (Figure 3E-F).

Then, since our team specializes in glioma, we focused on the relationship between PLK1 mRNA levels and prognosis of patients with glioma. The correlation between PLK1 mRNA levels and the prognosis of patients with pan-glioma, LGG, and GBM was investigated using the CGGA and TCGA datasets. In the CGGA-325, CGGA-693, and TCGA datasets, high PLK1 mRNA levels were associated with shorter OS in pan-glioma and LGG patients (Supplementary Figure 3A-B). However, the relationship between PLK1 mRNA levels and OS of patients with GBM was only statistically significant in CGGA-693 datasets (Supplementary Figure 3C). And the ROC curve verified that PLK1 could be an effective factor for predicting pan-glioma in the CGGA-325, CGGA-693, and TCGA datasets (Supplementary Figure 3D). Moreover, high PLK1 mRNA levels were associated with shorter disease specific survival (DSS) and progression free survival (PFI) in pan-glioma and LGG patients in TCGA dataset (Supplementary Figure 3E-F). In addition, multiple Cox regression revealed grade, IDH mutations, 1p/19q codeletions, promoter methylation of MGMT, and PLK1 mRNA levels might be independent predictors of prognosis of glioma patients (Supplementary Figure 4A). Similarly, the nomogram showed similar results (Supplementary Figure 4B). Therefore, we next explored the relationship between these molecular indicators and the prognosis of patients with glioma. As shown in Supplementary Figure 5A, patients in PLK1-high group had poorer prognosis compared to those in PLK1-low group in both IDH mutated and non-mutated glioma patients in CGGA-325 and CGGA-693 datasets. As shown in Supplementary Figure 5B, patients in PLK1-high group had poorer prognosis compared to those in PLK1-low group only in 1p19q non-codeletion glioma patients in CGGA-325, CGGA-693, and TCGA datasets. As shown in Supplementary Figure 5C, patients in PLK1-high group had poorer prognosis compared to those in PLK1-low group both

in MGMT promoter methylated and no-methylated glioma patients in CGGA-325, CGGA-693, and TCGA datasets. As shown in Supplementary Figure 5D and 5E, patients in PLK1-high group had poorer prognosis compared to those in PLK1-low group both in chemoradiotherapy and no- chemoradiotherapy glioma patients in CGGA-325 and CGGA-693 datasets.

Overall, the results demonstrated that the PLK1 mRNA levels were associated with the prognosis of multiple cancers. Moreover, higher PLK1 mRNA levels were associated with poorer prognosis of glioma patients.

3.4 Enrichment analysis of PLK1 related genes

In order to further explore the molecular mechanisms of PLK1 in tumorigenesis among pan-cancer, we mined PLK-binding proteins to conduct a protein-protein interaction network and the PLK1 expression-related genes to perform a battery of enrichment analyses. We obtained 50 PLK1-binding proteins with experimental verification based on the online website STRING. And the network diagram graphically showed the interactions of these proteins (Figure 4A). In order to determine the subcellular localization of the PLK1 proteins, we used the SangerBox database to investigate that the PLK1 proteins were mainly localized on the cytoskeleton of the cytoplasm (Figure 4B). Furthermore, we obtained a total of top 100 genes significantly positively correlated with PLK1 gene by GEPIA2 with TCGA database (Supplementary Table 3). Subsequently, we performed KEGG and GO-BP enrichment analyses using the top 100 positively correlated genes. The results of KEGG and GO-BP enrichment analyses are shown in Figure 4C-D. Our enrichment results showed that the top 100 genes were enriched not only in cell cycle-related pathways and terms but also in genetic alterations and immune related pathways and terms, such as “cell cycle”, “mismatch repair”, and “immune response” (Figure 4C-D).

In addition, we conducted enrichment analyses using PLK1 related genes in glioma. Analyses of PLK1 related genes in CGGA-325 and TCGA databases were performed using R package. Then, the heat map of PLK1-related genes was shown in Supplementary Figure 6A-B. And we performed KEGG and GO-BP enrichment analyses using the correlated genes (R>0.35 in CGGA-325; R>0.55 in TCGA) (Supplementary Figure 6A-B). Similarly, GSE67102 and GSE46856 databases were used to analyze the biological functions of PLK1 related genes in glioma. And the volcano plot, KEGG, GO-BP enrichment analyses of PLK1 related genes was analyzed and mapped in glioma by SangerBox portal (Supplementary Figure 7A-B). Like the results in pan-cancer, the results demonstrated that compared to glioma with low PLK1 expression, glioma with high PLK1 expression were enriched not only in classical carcinogenic signaling pathways and terms but also in cell cycle, genetic alterations, and immune related pathways and terms (Supplementary Figure 6-7).

Therefore, based on these analysis results, we speculated that PLK1 might promote tumor genesis and development by affecting cell cycle, genetic alterations, and antitumor immune in pan-cancer, especially in glioma.

3.5 Alterations of PLK1 gene are associated with development and progression of pan-cancer including glioma

Enrichment analysis of PLK1 related genes showed that PLK1might promote tumor genesis and development by affecting genetic alterations. Genetic alterations such as the mutations, deletions, or amplifications of oncogenes or tumor suppressor genes are associated with growth and progression of several tumors. Therefore, we first analyzed different types of alterations including mutations, structural variations, amplifications, and deep deletions in the PLK1 gene in using the TCGA cancer datasets with the cBioPortal portal. Among the 32 tumor types, PLK1 gene alteration frequency was the highest in UCEC cases (>5%), and the “mutation” type was dominant (Figure 5A). What is noteworthy is that all MESO (~1.0% frequency) and PAAD (~0.5% frequency) cases with gene alteration are “amplification” type (Figure5A). In addition, we discovered that the most abundant mutation type of PLK1 was “missense mutation” in pan-cancer (Figure 5B). R293H/C alteration in the Pkinase domain of PLK1 protein, which was discovered in 2 cases of COAD, 1 case of LUAD, 1 case of ESCA and 1 case of HNSC, can lead to missense mutation of the PLK1 gene, translating from R (Arginine) to H (Histidine) or C (Cysteine) at the site 293 of PLK1 protein, and changing the structure of PLK1 protein subsequently (Figure 5B). The genetic alteration effect of R293H/C was displayed in the 3D structure of PLK1 protein (Figure 5C).

In addition, to determine whether PLK1 expression levels were associated with specific genomic characteristics in gliomas, we performed copy number variation (CNV) and somatic mutation analysis using the TCGA dataset. A distinct overall CNV profile emerged from the comparison of the PLK1-low (n = 170) versus the PLK1-high (n = 170) cluster (Figure 5D-F). Co-deletion of 1p and 19q, a genomic hallmark of oligodendroglioma, more frequently appeared to be associated with the PLK1-low cluster (Figure 5D). Amplification of chr7 and deletion of chr10, which are both common genomic events in GBM, frequently occurred in the PLK1-high cluster (Figure 5D). The comparison of the CNV profiles in the PLK1-low (n = 170) and PLK1-high (n = 170) samples is shown in Figure 5E-F. In PLK1-high group, frequently amplified genomic regions included oncogenic driver genes, such as EGFR (7p11.2), IK3C2B (1q32.1), PDGFRA (4q12), and CDK4 (12q14.1), whereas deleted regions contained tumor suppressor genes, including CDKN2A/CDKN2B (9p21.3), PARK7 (1p36.23), and PTEN (10q23.3). In PLK1-low samples, significant amplifications showed peaks in 7p11.2, 8q24.13, 12p13.3, and 19p13.3, whereas the frequently deleted genomic regions were 2q37.3, 9p21.3, 13q21.33, and 14q23.2. The PLK1-low group (n = 170) showed high frequency of somatic mutations in the IDH1 (75%), TP53 (36%), ATRX (32%), and CIC (28%) genes and the PLK1-high group (n = 170) showed high frequency of mutations in the TP53 (38%), TTN (33%), PTEN (31%), and EGFR (30%) genes (Figure 5G-H).

Overall, these results showed PLK1 gene mutations, amplifications, and deletions in multiple tumors and missense mutations were the most frequent type. Moreover, the glioma tissues showed distinct somatic mutations and CNVs based on the expression levels of PLK1. These results suggested that the alteration of PLK1 gene might be a potential mechanism to lead to the occurrence and development of various tumors, especially glioma.

3.6 PLK1 expression is associated with the antitumor immunity in pan-cancer including glioma

Enrichment analysis of PLK1 related genes implied that PLK1 might promote tumor genesis and development by affecting antitumor immune in pan-cancer. Therefore, we also analysis the relationship between PLK1 expression and the tumor immune microenvironment (TIM) in pan-cancer, especially in glioma.

First, we evaluated the correlation between ESTIMATE scores (ESTIMATE, immune, and stromal scores) and PLK1 mRNA levels in pan-cancer. Immune score reflects the proportion of infiltrated immune cells in the tumor tissues; stromal score reflects the proportion of stromal cells in the tumor tissues. ESTIMATE score is the sum of immune and stromal scores, and reflects the status of the tumor immune microenvironment and tumor purity. Our results demonstrated negative correlation between PLK1 mRNA levels and the ESTIMATE, immune, and stromal scores in glioblastoma multiforme (GBM), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), uterine corpus endometrial carcinoma (UCEC), testicular germ cell tumors (TGCT), esophageal carcinoma (ESCA), pancreatic adenocarcinoma (PAAD), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), sarcoma (SARC), stomach adenocarcinoma (STAD), skin cutaneous melanoma (SKCM), head and neck squamous cell carcinoma (HNSC), and rectum adenocarcinoma (READ) (Figure 6A). This suggested that high PLK1 mRNA levels were associated with decreased infiltration of immune and stromal cells in these tumors. Similarly, PLK1 mRNA levels showed negative correlation with ESTIMATE, immune, and stromal scores in THCA (Figure 6A). Supplementary Figure 8 showed the negative correlations between PLK1 mRNA levels and ESTIMATE score, Immune score and Stromal score in GBM.

Tumor-infiltrating immune (TIIs) cells, as an important part of TIM, are usually related to the occurrence, progression, treatment, or metastasis of tumor. Moreover, many reports have claimed that tumor-infiltrating lymphocytes (TILs) are critical predictors of sentinel lymph node status and survival in cancers[44]. Thus, we then used the online tool Sangerbox and TISIDB to analysis the relationship between abundance of TIIs/TILs and PLK1 mRNA levels. PLK1 mRNA levels showed negative correlation with multiple TIIs/TILs in GBM (Figure 6B-C). Supplementary Figure 9 showed the relationship between PLK1 mRNA levels and various TIIs/TILs levels in GBM in CGGA-325, CGGA-693, and TCGA datasets using the ImmuCellAI. The heat maps of the infiltration levels of various TIIs/TILs in GBM were shown in Supplementary Figure 10.

The immune checkpoint (ICP) blockade proteins are the most promising targets of cancer immunotherapeutic treatments. Therefore, we analyzed the relationship between expression levels of ICP genes and PLK1 in multiple cancer types. PLK1 mRNA levels showed negative correlation with ICP genes in GBM (Figure 6D). These results suggested that PLK1 might affect the sensitivity of GBM to the immune checkpoint inhibitor therapies. Next, we analyzed the correlation between PLK1 and various ICP receptors and ligands in GBM patients from the CGGA-325, and TCGA datasets. PLK1 mRNA levels showed association with ICP receptors and ICP ligands in GBM tissues (Supplementary Figure 11). Therefore, we hypothesized that PLK1 might alter the immune microenvironment in the GBM tissues by

modulating the expression levels of ICP receptors and ligands. These results suggested that PLK1 mediated the activation of ICP genes and was an ideal target for immunotherapy of GBM patients.

In addition, we also investigated the effects of PLK1 mRNA levels on the TIM of GBM by screening seven metagenes, namely, HCK, IgG, Interferon, lymphocyte-specific kinase (LCK), MHC-I, MHC-II, and STAT1, which reflect the status of inflammation and immune responses. ***HCK***: This cluster encompasses genes specific for macrophages and cells of the monocyte/myeloid lineage; ***IgG***: Most of the genes in this cluster represent genes of immunoglobulins of the immunoglobulin gamma type mainly associated with B lymphocytes; ***Interferon***: All genes in this cluster represent genes known to be interferon inducible and that are associated with the interferon response of cells; ***LCK***: Genes in this cluster contain T-cell-specific markers; ***MHC-I***: This cluster contains HLA-A, HLA-B, HLA-C, HLA-F and HLA-G genes of the major histocompatability class I complex common to all cell types for the presentation of intracellular antigens; ***MHC-II***: This cluster contains the HLA-DP, HLA-DQ, HLA-DR genes of the major histocompatability class II complex expressed on the surface of professional antigen-presenting cells for their interaction with T cells; ***STAT1***: The genes in this cluster are associated with interferon signal transduction and are also interferon inducible[45]. Our results showed that PLK1 mRNA levels were negatively correlated with enrichment scores of Interferon (All genes in this cluster represent genes known to be interferon inducible and that are associated with the interferon response of cells) and LCK (Genes in this cluster contain T-cell-specific markers) (Supplementary Figure 12). This suggested that PLK1 might regulate interferon signaling and T cell signaling in GBM.

Overall, our results suggested that PLK1 could regulate the TIM and modulate the sensitivity of several tumors to immunotherapy. Therefore, PLK1might be a potential immunotherapy biomarker and predictor of tumor immunotherapeutic response.

3.7 Experimental verification of PLK1 expression and phenotype in glioma

Although the results of a series of bioinformatics analyses had confirmed that PLK1 played oncogenic roles in pan-cancer, the experimental verification was more convincing. Therefore, we demonstrated the expression differences and biological roles of PLK1 in normal human astrocyte (HA) cell lines and several glioma cells lines through some experiments, taking glioma as the representative. Moreover, we confirmed the high expression of PLK1 in glioma tissues by RNA sequencing of 100 glioma tissues.

The qRT-PCR showed that PLK1 RNA expression levels in glioma cell lines were significantly higher than that in NHA cells, and the highest expression levels were found in U87 cells (Figure 7A). Moreover, analysis of 100 glioma cases collected also showed that PLK1 mRNA level was positively correlated with glioma grade and poorer prognosis, which was consistent with our analysis results in CGGA and TCGA databases (Figure 7B-C).

Subsequently, we selected the two cell lines (U87 and LN229) to conduct experiments related to cell cycle and proliferation in order to verify the results of enrichment analyses. The PLK1 gene in two glioma cell lines was knocked down with siRNA. Its expression was then confirmed across qRT-PCR (Supplementary

Figure 13). The flow cytometry analyses showed that the percentage of cells in G2/M phase was increased in si-PLK1 group compared to the control group (Figure 7D-E). These results suggested that si-PLK1 could induce G2/M arrest. Furthermore, the CCK8 and EdU assays showed that knockdown of PLK1 led to significantly decreased cell proliferation (Figure 7F-I).

In conclusion, we took glioma as an example to verify the conclusions of the above bioinformatics analysis through experiments. That is, PLK1 played important roles in tumor pathology by regulating cell cycle and cell proliferation.

3.8 The DNA methylation levels of PLK1 in different human cancers

In order to study the mechanism of abnormal expression of PLK1, we also performed PLK1 DNA methylation analysis. DNA methylation of oncogenes usually enhances their expression level and leads to tumor development [46].

The online tool UALCAN was used to explore the methylation level in the PLK1 promoter region. Similar to the above results, PLK1 promoter methylation levels were lower in thyroid carcinoma (THCA), uterine corpus endometrial carcinoma (UCEC), lung adenocarcinoma (LUAD), rectum adenocarcinoma (READ), bladder urothelial carcinoma (BLCA), liver hepatocellular carcinoma (LIHC), esophageal carcinoma (ESCA) and testicular germ cell tumors (TGCT) compared to the normal tissues (Supplementary Figure 14). These results may imply that the promoter methylation of PLK1 might contribute its abnormal expression.

Through the analysis of online tool MEXPRESS, we observed that the PLK1 mRNA expression levels were negatively related to the PLK1 methylation levels in both LGG and GBM (Figure 8A-B). The PLK1 mRNA levels were negatively related to the PLK1 methylation levels at probe ID: cg04138181 ($r=-0.274$, $P<0.001$) and probe ID: cg04758185 ($r=-0.110$, $P<0.05$) in LGG (Figure 8A). The mRNA expression levels of PLK1 were negatively related with the methylation levels of PLK1 at probe ID: cg05657488 ($r=-0.373$, $P<0.01$) and probe ID: cg04138181 in GBM ($r=-0.267$, $P<0.05$) (Figure 8B). Additionally, the relationship between PLK1 methylation levels and WHO grade of glioma was analyzed across the CGGA database. We also used the CGGA database to analyze the correlation between PLK1 methylation levels and survival of glioma patients. The results indicated that the levels of PLK1 methylation were negatively associated with the WHO grade of glioma. The methylation levels of PLK1 in WHO gliomas were significantly higher than that in WHO and WHO gliomas (Figure 8C). In the survival analysis of primary glioma samples, the lower levels of PLK1 methylation were associated with poorer prognosis (Figure 8D).

In summary, these results suggested that low methylation levels of PLK1 might contribute to its overexpression in pan-cancer, especially in glioma.

3.9 Construction of the upstream lncRNA-miRNA regulatory network that regulates PLK1 expression in glioma and other tumors

In recent years, several studies have shown that long non-coding RNAs (lncRNAs) play a significant role in tumorigenesis by regulating the expression of the downstream mRNAs through sequestering of their target miRNAs. Therefore, we investigated the lncRNA-miRNA network that may regulate PLK1 expression in various tumors especially in glioma. First, we screened the miRWalk, TargetScan, and miRmap databases and identified 47 miRNAs that potentially target the PLK1 mRNAs (Figure 9A). The top 10 PLK1 mRNA-targeting miRNAs were hsa-miR-296-5p, hsa-miR-92a-2-5p, hsa-miR-3665, hsa-miR-4660, hsa-miR-1185-1-3p, hsa-miR-1185-2-3p, hsa-miR-509-3-5p, hsa-miR-509-5p, hsa-miR-3120-3p and hsa-miR-4728-5p (Figure 9B).

Among these 10 PLK1 mRNA-targeting miRNAs, 4 miRNAs (hsa-miR-296-5p, hsa-miR-92a-2-5p, hsa-miR-509-3-5p, and hsa-miR-509-5p) were found in the CGGA database. Then, we analyzed the relationship between these 4 miRNAs expression levels and prognosis and glioma grades in CGGA dataset (Figure 9C-D and Supplementary Figure 15). The results showed that hsa-miR-296-5p and hsa-miR-92a-2-5p expression levels had statistic relationship with both prognosis and grades. Moreover, hsa-miR-92a-2-5p expression level associated with better prognosis and the hsa-miR-92a-2-5p expression level was negatively associated with glioma grades, which implied that hsa-miR-92a-2-5p was tumor suppressor in glioma. Therefore, these results suggested that hsa-miR-92a-2-5p potentially targeted PLK1 mRNA in glioma.

Next, we identified lncRNAs that may target hsa-miR-92a-2-5p using the TargetScan database. The top 10 predicted lncRNAs and top 10 validated lncRNAs were used to construct a lncRNA-miRNA-PLK1 regulatory network using the cytoscape software (Figure 9E). These results demonstrated the upstream lncRNA-miRNA regulatory network that may regulate the aberrant expression of PLK1 in the glioma.

Discusion

PLKs, in mammals, has diverged into five paralogues, PLK1-5. It has been widely reported that PLK1 is a member of PLK kinases family and plays important roles in cell cycle and mitosis[47]. A large number of newly-presented literatures have reported that aberrant expression of PLK1 may lead to many clinical diseases, especially cancers[48]. Whether PLK1 can exert effects in the pathological process of various cancers through some similar molecular mechanisms has yet to be explored. By searching literatures, we found that there was little analysis of the oncogenic roles of PLK1 in pan-cancer. Therefore, we used a number of databases to detect the molecular features of PLK1 gene expression, gene alteration, immune infiltration, cell cycle in pan-cancer, especially glioma. What's more, we also explored the potential molecular mechanisms of PLK1 aberrant expression by analyzing methylation of PLK1 DNA and ceRNA network in glioma.

Firstly, we explored the expressions of PLK1 in various cancers and normal tissues since many previous studies have claimed that abnormal expressions of PLK1 can attract the occurrence of numerous diseases, including cancers[49]. PLK1 was highly expressed in both mRNA and protein levels in several tumors (Fig. 1–2). The pan-cancer analysis also showed statistic correlation between PLK1 expression

level and the prognosis (Fig. 3). Since our specialty was neuro-oncology, we subsequently conducted a detailed study on the relationship between PLK1 expression and prognosis in glioma. In our studies, high PLK1 level was also associated with clinical features such as grade, IDH mutation status, 1p/19q co-deleted status, and methylation status of MGMT promoter in glioma (Supplementary Fig. 2). RNA-seq of 100 glioma cases in our sample database discovered that glioma patients with high PLK1 level had poorer prognosis compared with patients with low PLK1 level (Fig. 7D), which was consistent with the results using CGGA and TCGA datasets (Supplementary Fig. 3A). Cox regression analysis showed that PLK1 was an independent prognostic predictor in glioma (Supplementary Fig. 4). Recent research reported that aberrant upregulated PLK1 correlates with recurrence and poor prognosis in colorectal cancer patients[50]. Qian et al validated that PLK1 was highly expressed in clear cell renal cell carcinoma (ccRCC) tissues and promoted ccRCC cell proliferation, migration, invasion, and cell cycle[51]. He et al reported that PLK1 was highly expressed and predicted a poor prognosis in hepatocellular carcinoma patients[52]. Similarly, Wang et al demonstrated that PLK1 levels were elevated in glioma compared with those in normal brain tissues, and high expression of PLK1 was associated with poor prognosis[53]. Overall, our analysis showed that PLK1 was highly expressed and a potential prognostic biomarker in various cancers, especially in glioma.

Secondly, in order to explore the oncogenic mechanisms of PLK1 in pan-cancer, the top 100 PLK1 positively correlated genes in pan-cancer were obtained to perform enrichment analysis (Fig. 4). Consistent with the results in pan-cancer, PLK1-related genes were enriched not only in classical carcinogenic signaling pathways and terms but also in cell cycle, genetic alteration and immune related pathways and terms in glioma (Supplementary Fig. 6–7). Therefore, we mainly explored the molecular mechanism of PLK1 exerting oncogenic effects by regulating cell cycle, genetic alteration and immune process in detail.

PLK1 gene alteration was the most frequent in UCEC patients among 32 cancer types, and was mainly represented as mutation. Notably, missense mutation was the most abundant form of PLK1 gene alteration, and it mainly manifested as the mutation at the 293 site of PLK1 protein from R(Arginine) to C(Cysteine)/H(Histidine) (Fig. 5A-C). Gao et al reported that the predominant type of mutation for PLK1 was missense mutation in cervical cancer, which consistent with the result of our pan-cancer analysis. In addition, since the R293C/H missense mutation occurred in the Pkinase domain of PLK1 protein, we speculated that the R293C/H missense mutation could lead to the genesis and progression of cancers by changing the Pkinase activity of PLK1 proteins. Nevertheless, we have not found the R293C/H missense mutations of PLK1 in previous studies. Therefore, the R293C/H missense mutation of PLK1 may serve as a new potential direction for tumor research in the future. Next, we explored the correlation between gene alterations of characteristic molecules and PLK1 expression level in glioma. Isocitrate dehydrogenase (IDH) mutation and chromosomal 1p/19q codeletions are associated with better survival outcomes of glioma patients. The chromosomes 7/10 were also molecular profiles characteristically altered in glioblastoma multiforme (GBM) according to the 2021 WHO classification of CNS tumors[54]. In brief, amplification of chromosome 7 and deletion of chromosome 10 predicts poor prognosis in patients with glioma. The co-deletion of 1p/19q was significant, and the IDH mutation rate was up to 75% in glioma

with PLK1 low expression. The amplification of chromosome 7 and deletion of chromosome 10 were significant in glioma with PLK1 high expression with high mutation rate of TP53 (38%) (Fig. 5D-H). These results suggest that both the variation of PLK1 itself and other gene variations related to PLK1 expression have an impact on the occurrence and progression of tumors, especially glioma.

Following, increasing literatures showed that immune effects played critical roles in anti-tumor mechanisms and may serve as new diagnostic and therapeutic potential targets in cancers. Several studies have shown that tumor-infiltrating immune cell/tumor-infiltrating lymphocytes (TIIs/TILs) are important components of the tumor microenvironment. The immune checkpoint (ICP) proteins are the most promising targets of cancer immunotherapeutic treatments and they can regulate the bioactivities of immune cells in the tumor microenvironment. Moreover, immunity/inflammatory-related metagenes can help tumor to obtain multiple hallmarks and regulate the tumor progression. Therefore, the association between PLK1 gene expression levels and tumor microenvironment was analyzed. The results of pan-cancer analysis implied that PLK1 expression levels were negatively corelated with the multiple TIIs/TILs levels in several cancer types (Fig. 6B-C). Similarly, Takeshita et al found that PLK1 mRNA expression was significantly associated with CD8 + T cells, activated memory CD4 + T cells, M0 macrophage, M1 macrophage, and M2 macrophage in ER positive Her2 negative breast cancer[55]. And Park et al claimed that PLK1 could be a universal tumor antigen recognized by cytotoxic T lymphocytes for cancer immunotherapy in murine tumor models[56]. ESTIMATE analysis showed that the expression of PLK1 negatively correlated with the infiltration of immune cells and stromal cells in glioblastoma multiforme (GBM) (Supplementary Fig. 8). Similarly, PLK1 expression levels were negatively corelated with infiltration levels of multiple immune cells in glioblastoma multiforme (GBM) in CGGA and TCGA datasets using ImmuCellAI (Supplementary Fig. 9). These results suggested that PLK1 inhibited the infiltration of immune cells into glioblastoma multiforme (GBM), thereby enabling the tumor cells to evade the immune system. Zhou et al found that inhibiting PLK1 could alter the tumor immune microenvironment by enriching T cells infiltration non-small cell lung cancer (NSCLC), which was consistent with our conclusion[57]. Immune check point (ICP) proteins play a significant role in the tumor-infiltration of immune cells and immunotherapy. The results of pan-cancer analysis using SangerBox implied that PLK1 expression levels were negatively corelated with the several ICP genes levels in glioblastoma multiforme (GBM) and testicular germ cell tumors (TGCT), but positively associated with several ICP genes levels in liver hepatocellular carcinoma (LIHC), cholangiocarcinoma (CHOL), kidney renal clear cell carcinoma (KIRC), thyroid carcinoma (THCA), and kidney chromophobe (KICH) (Fig. 6D). Consistent with the results of pan-cancer, PLK1 expression negatively correlated with the expression of multiple ICP genes in glioblastoma multiforme (GBM) in CGGA and TCGA datasets (Supplementary Fig. 11). PLK1/vimentin signaling facilitates immune escape by upregulating PD-L1 in metastatic lung adenocarcinoma[58]. In addition, inflammation can help to obtain multiple hallmarks by providing bioactive molecules to the tumor microenvironment and can promote the development of early tumors into mature cancers[59]. Our results about immunity/inflammatory-related metagenes showed that PLK1 mRNA levels were negatively correlated with enrichment scores of Interferon and lymphocyte-specific kinase (LCK) in glioblastoma multiforme (GBM) (Supplementary Fig. 12), which implied that Interferon

and LCK might be protective factors, different from PLK1. A previous study reported that “Immunity” metagene was associated with a better prognosis in HER2-positive/ER-negative breast cancers, which was consistent with our results[60]. Callari et al claimed that the IFN metagene was associated with a low risk of metastasis in 104 ERBB2 + tumors[61]. Similarly, Ma et al confirmed that the survival rate of patients with high LCK metagene expression was markedly higher than that of the low expression group in the endometrioid endometrial adenocarcinoma subtype group[62].

Furthermore, qRT-PCR has verified that PLK1 is high expressed in glioma tissues and cell lines (Fig. 7A). And flow cytometry, CCK8 and EdU assays have verified that PLK1 can accelerate cell cycle and stimulate cell proliferation in glioma cell lines (Fig. 7D-I). Similarly, Wu et al confirmed that of PLK1 significantly promoted cell proliferation, migration, invasion, and inhibited apoptosis of U87 and U251 glioma cells[63]. Cheng et al reported that down-regulation of PLK1 could inhibit growth, induce cell arrest in G2/M phase of cell cycle and apoptosis enhancement in glioma cells[64]. Interestingly, some studies have shown that PLK1 inhibitors could inhibit glioma cell proliferation and glioma progression. Such as, Li et al reported that BI2536 (PLK1 inhibitor) could diminish glioma stem cells (GSC) self-renewal in vitro, and increase survival of orthotopic tumor-bearing mice[65].

Lastly, to explore possible molecular mechanisms of aberrant expressions of the PLK1, we attempted to analyze whether PLK1 DNA methylation and ceRNA network could affect PLK1 expression levels. It has been reported that gene methylation often leads to low expression levels. Compared with normal tissue, the PLK1 gene promoter region was less methylation in several tumors (Supplementary Fig. 14). Taking glioma as an example, the methylation level of PLK1 was negatively correlated with PLK1 expression level (Fig. 8A-B). Furthermore, high methylation levels of PLK1 contributed to poor prognosis and advanced grades in glioma patients (Fig. 8C-D). Consistent with our analysis, a previous study reported PLK1 which was typically hypermethylated in normal liver tissue but became hypomethylated and upregulated in liver tumor[66]. In addition, we also conducted a lncRNA-miRNA regulatory network that may regulate the aberrant expression of PLK1 in glioma (Fig. 9). The analysis results suggested that the gene methylation and ceRNA regulatory network of PLK1 might be the important molecular mechanisms that contribute to the aberrant expressions of PLK1.

In summary, our results suggested that PLK1 was overexpressed in various cancers and significantly correlated with the poorer prognosis. The results of bioinformatics analysis indicated that gene alteration and anti-tumor immunity might be the potential oncogenic mechanisms of PLK1 in pan-cancer, especially glioma. In vitro experiments confirmed that PLK1 promoted glioma progression by regulating cell cycle. The analysis results implied that hypo-methylation of PLK1 and abnormal regulation of ceRNA network are responsible for its abnormal expression. In conclusion, our present study suggested that PLK1 may have potential as a diagnosis and prognostic marker as well as therapeutic target for several malignant tumors, especially glioma.

Abbreviations

ACC: Adrenocortical carcinoma; BLCA: Bladder Urothelial Carcinoma; BRCA: Breast invasive carcinoma; CHOL: Cholangiocarcinoma; COAD: Colon adenocarcinoma; DLBC: Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; 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; MESO: Mesothelioma; OV: Ovarian serous cystadenocarcinoma; PAAD: Pancreatic adenocarcinoma; PRAD: Prostate adenocarcinoma; READ: Rectum adenocarcinoma; SARC: Sarcoma; SKCM: Skin Cutaneous Melanoma; STAD: Stomach adenocarcinoma; TGCT: Testicular Germ Cell Tumors; THCA: Thyroid carcinoma; THYM: Thymoma; UCEC: Uterine Corpus Endometrial Carcinoma; UVM: Uveal Melanoma; OS: overall survival; DFS: disease-free survival; DSS: disease-specific survival; PFS: progression-free survival

Declarations

Data Availability Statement

Publicly available datasets were analyzed in this study. All data generated or analyzed during this study are included in this published article and its supplementary information files.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

LH, XL, and ZZ conceived the review. LL, XZ and YZ drafted the manuscript and revised it before submission. XL, DP, and ZZ collected the references. LL, LH and XZ performed the experiments. YZ, CW, and ZL contributed reagents/materials/analysis tools.

All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the National Natural Science Foundation of China (No. U1804172 to Xianzhi Liu, 81773187 to Lei Han and 81702465 to Zhenyu Zhang).

Acknowledgments

We acknowledge and appreciate our colleagues for their valuable efforts and comments on this paper.

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Figures

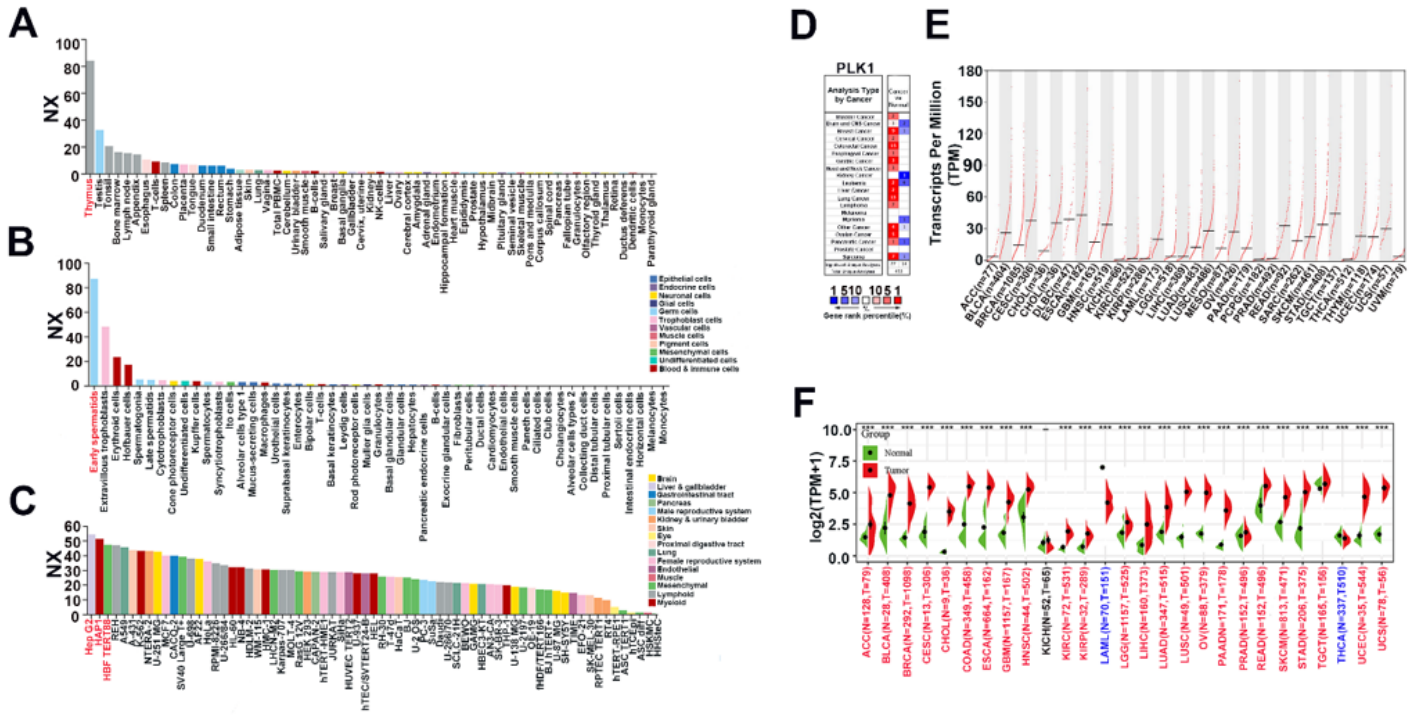

Figure 1

The expression pattern of PLK1 mRNA in pan-cancer.

(A). The expression status of the PLK1 mRNA in different human tissues were analyzed through HPA. (B). The expression status of the PLK1 mRNA in different human cells were analyzed through HPA. (C). The expression status of the PLK1 mRNA in different tumor cell lines were analyzed through HPA. (D). Increased or decreased PLK1 mRNA in datasets of different cancers compared with normal tissues in the Oncomine database (P<0.001, FC>1.5, gene rank=all). (E). PLK1 mRNA expression levels in different tumor types from TCGA database were determined by GEPIA2. (F). PLK1 mRNA expression levels in different tumor and normal tissues from TCGA and GTEx database were determined by SangerBox (*P<0.05, **P<0.01, ***P<0.001).

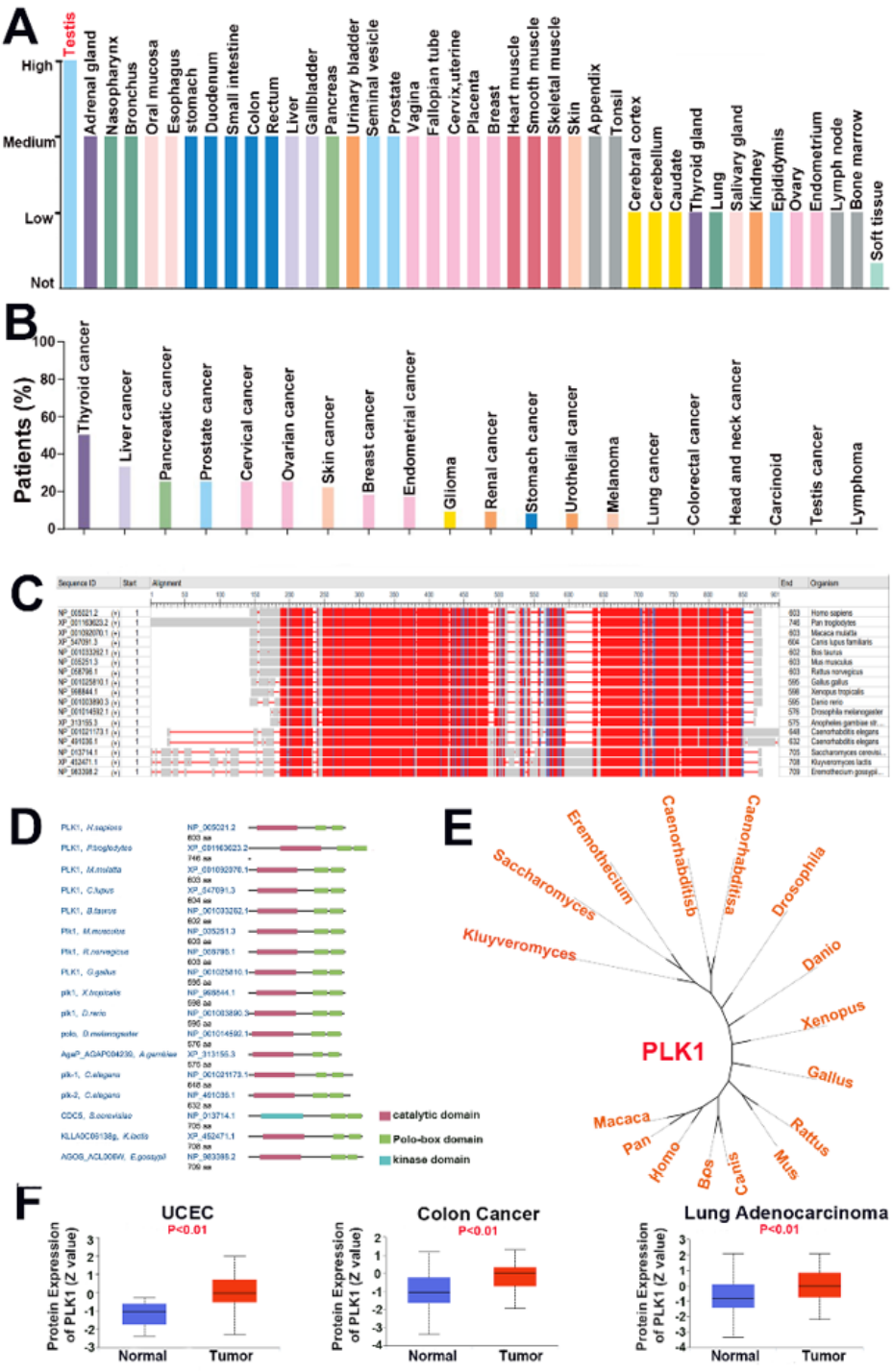

Figure 2

The expression pattern of PLK1 protein in pan-cancer.

The expression status of the PLK1 protein in different human normal (A) and cancer (B) tissues were analyzed through HPA. The expression status of the PLK1 protein in different cancer tissues were analyzed through HPA. (C). The conserved analysis of PLK1 amino acid among different species by

NCBI. (D). The conserved analysis of PLK1 domain among different species by NCBI. (E). The phylogenetic tree data presents the evolutionary relationship of the PLK1 protein across different species. (F). The differential expression of PLK1 protein in normal tissues and tumors in UCEC, colon cancer and lung adenocarcinoma across the CPTAC database of UALCAN online tool.

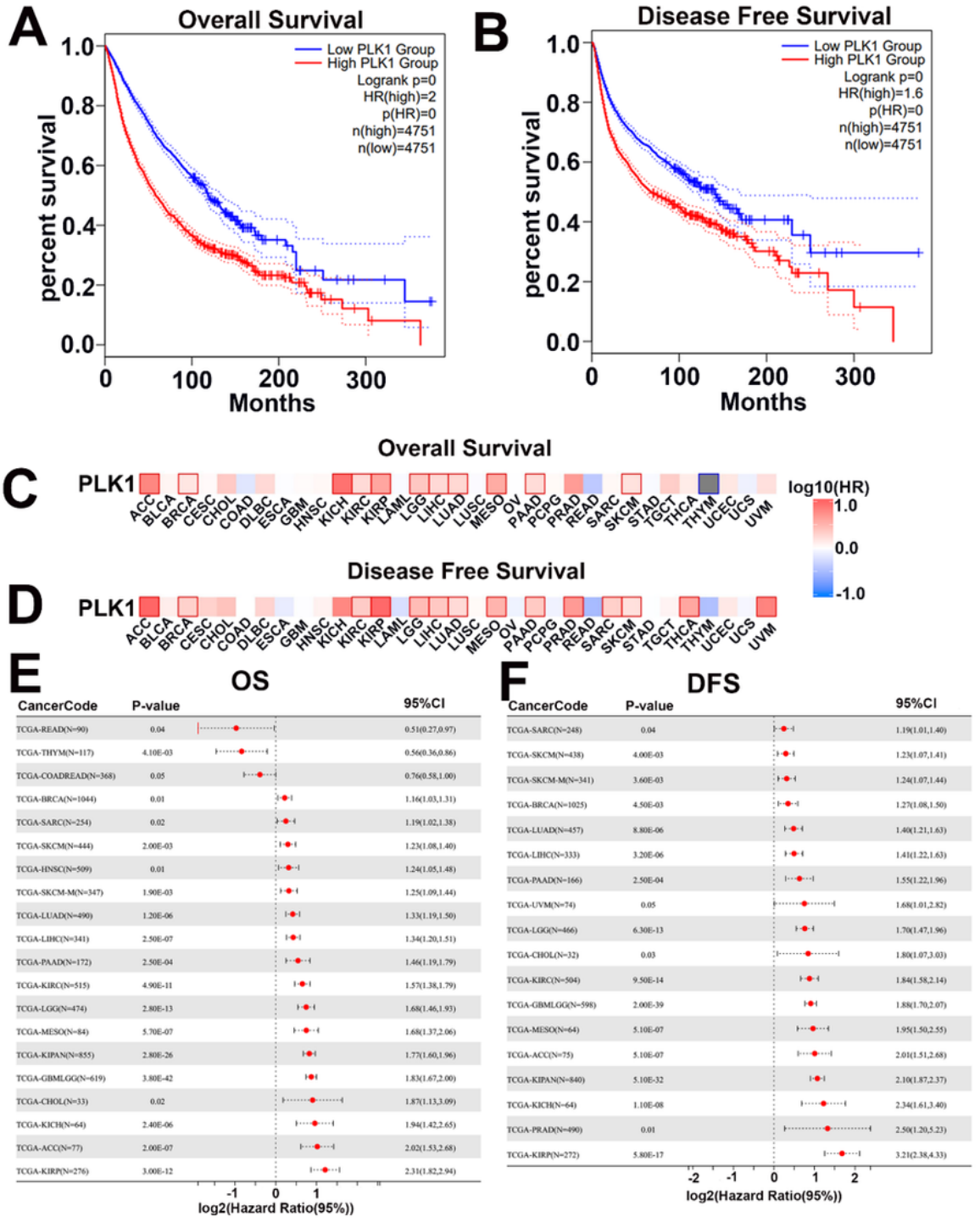

Figure 3

The prognostic potential of PLK1 mRNA expression levels in pan-caner.

The overall survival (A) and disease-free survival (B) analyses of pan-cancer in TCGA about PLK1 mRNA level using GEPIA2. The overall survival (C) and disease-free survival (D) analyses of different tumors in TCGA about PLK1 mRNA level using GEPIA2. (E). Relationship between PLK1 mRNA level and Cox-OS of different cancers in TCGA datasets using SangerBox. (F). Relationship between PLK1 mRNA level and Cox-DFS of different cancers in TCGA datasets using SangerBox.

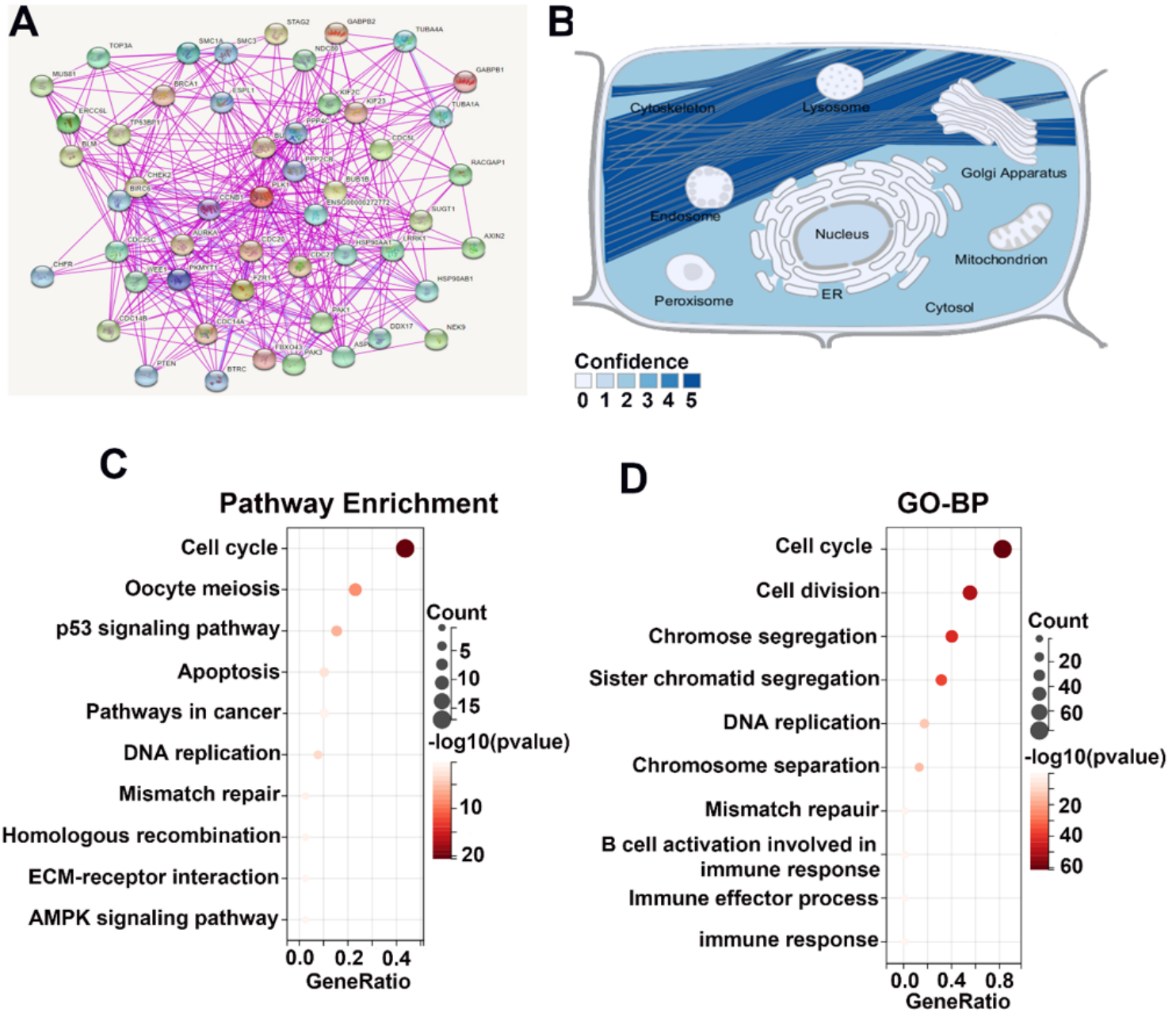

Figure 4

Enrichment analysis of PLK1-related genes.

(A). The experimentally determined PLK1-binding proteins were obtained using the STRING tool. (B). The simulation of the intracellular localization of PLK1 protein. The KEGG (C) and GO-BP (D) enrichment analyses were applied with the top 100 positively genes correlated with PLK1 expression in pan-cancer.

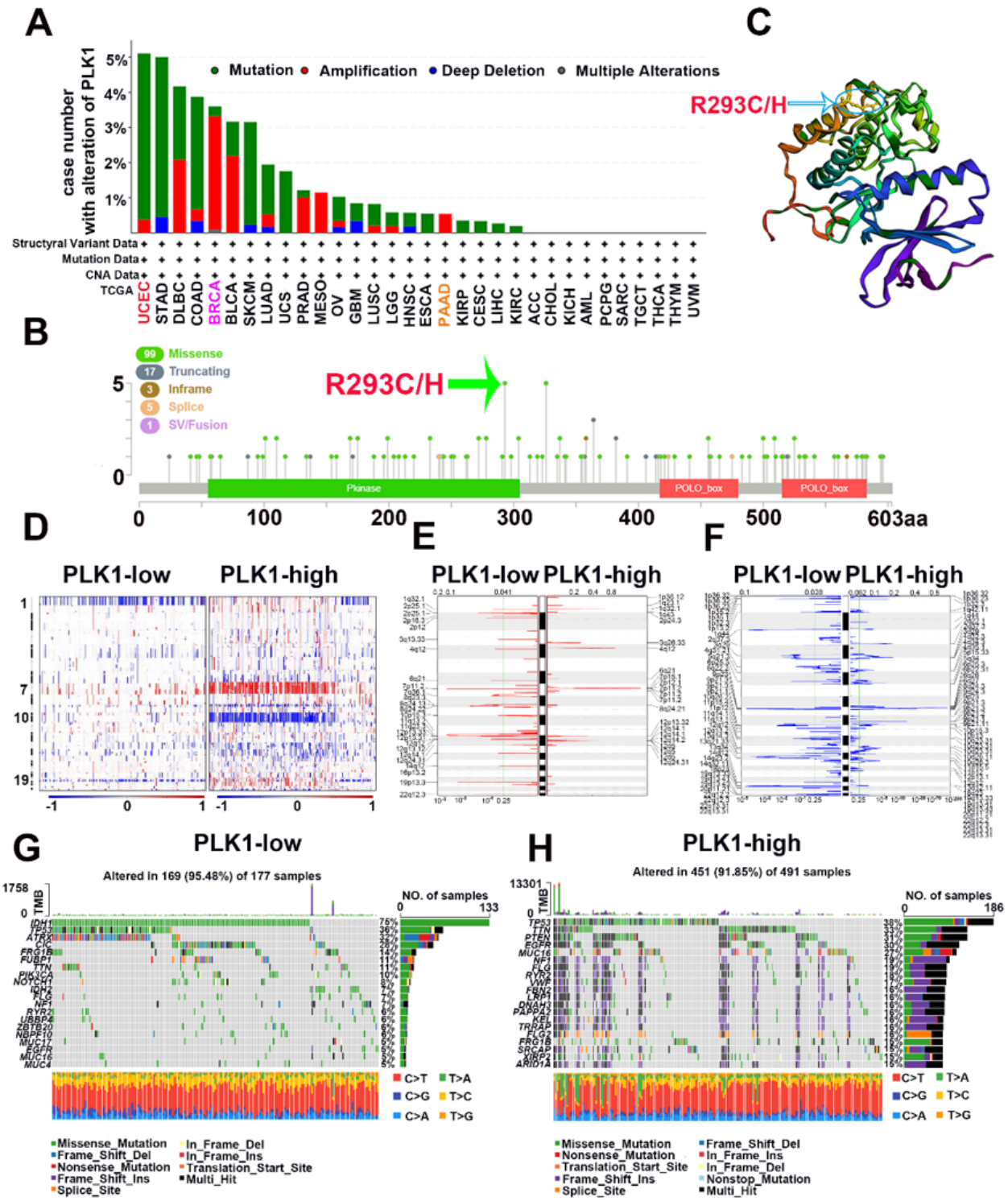

Figure 5

The DNA alterations of PLK1 in pan-cancer.

The alteration frequency with mutation type (A) and mutation site (B) are displayed using the cBioPortal tool in pan-cancer. The mutation site with the highest alteration frequency in the 3D structure of PLK1 is presented across the cBioPortal tool in pan-cancer (C). (D). Overall copy number variation (CNV) profile according to high vs low PLK1 expression. Blue (deletion); red (amplification). (E). Frequency of specific changes based on PLK1-low (left row) and PLK1-high (right row) groups. The Y-axis represents the frequency of chromosomal amplification (red). (F). Frequency of specific changes based on PLK1-low (left row) and PLK1-high (right row) groups. The Y-axis represents the frequency of chromosomal deletion (blue). (G). Spectrum of somatic mutations in gliomas from PLK1-low groups. (H). Spectrum of somatic mutations in gliomas from PLK1-high groups.

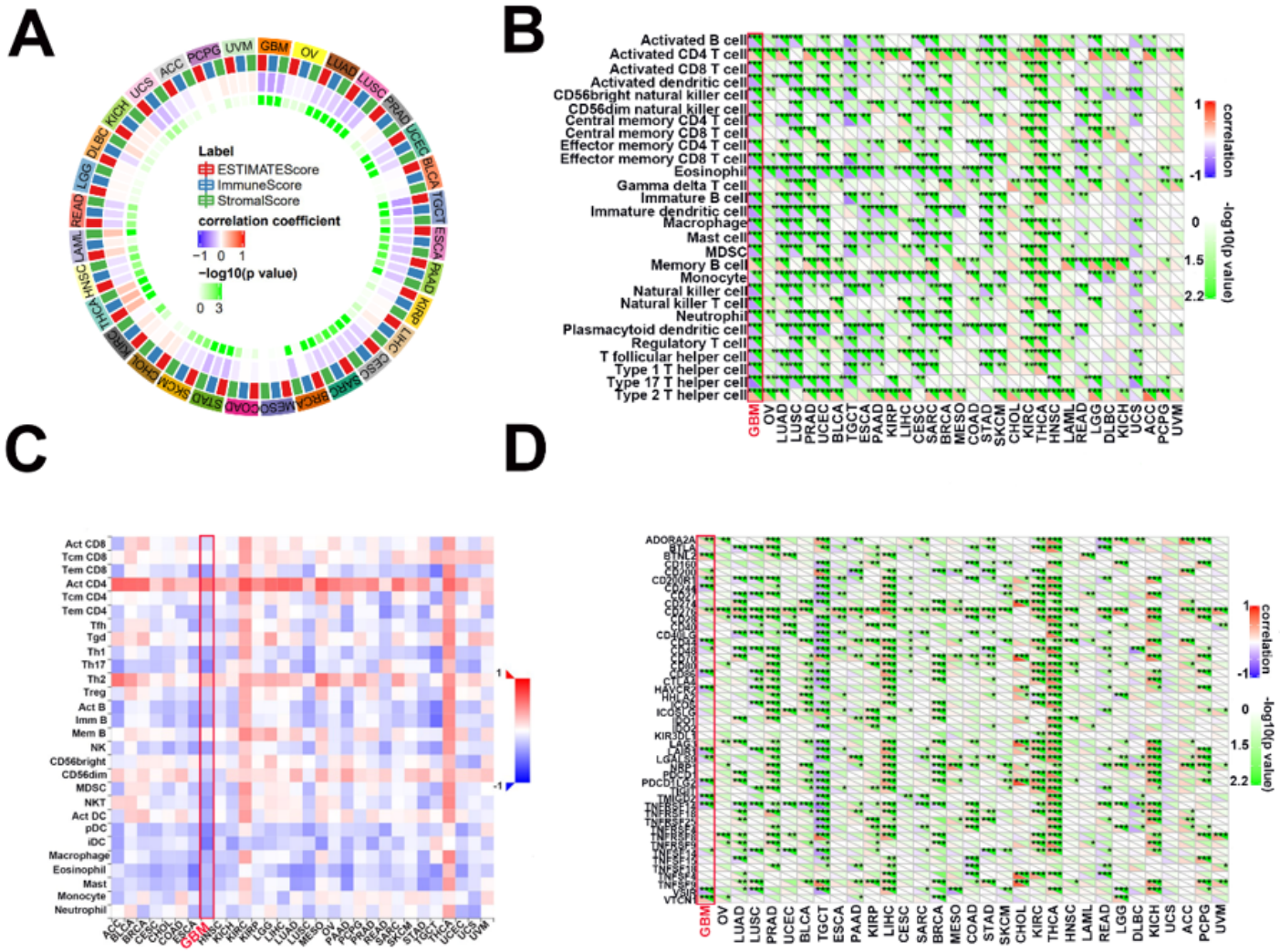

Figure 6

The relationship between the PLK1 mRNA levels and antitumor immune in pan-cancer.

(A). The correlations between ESTIMATE scores (ESTIMATE Score, Immune Score, and Stromal Score) and PLK1 mRNA levels were analyzed in various tumors by SangerBox portal. (B). The relationship between PLK1 mRNA levels and immune cell infiltration levels was analyzed in various tumors by SangerBox. (C). The relationship between PLK1 mRNA levels and abundance of tumor-infiltrating lymphocytes (TILs) using TISIDB. (D). The relationship between PLK1 mRNA levels and immune check point (ICP) genes was analyzed in various tumors by SangerBox.

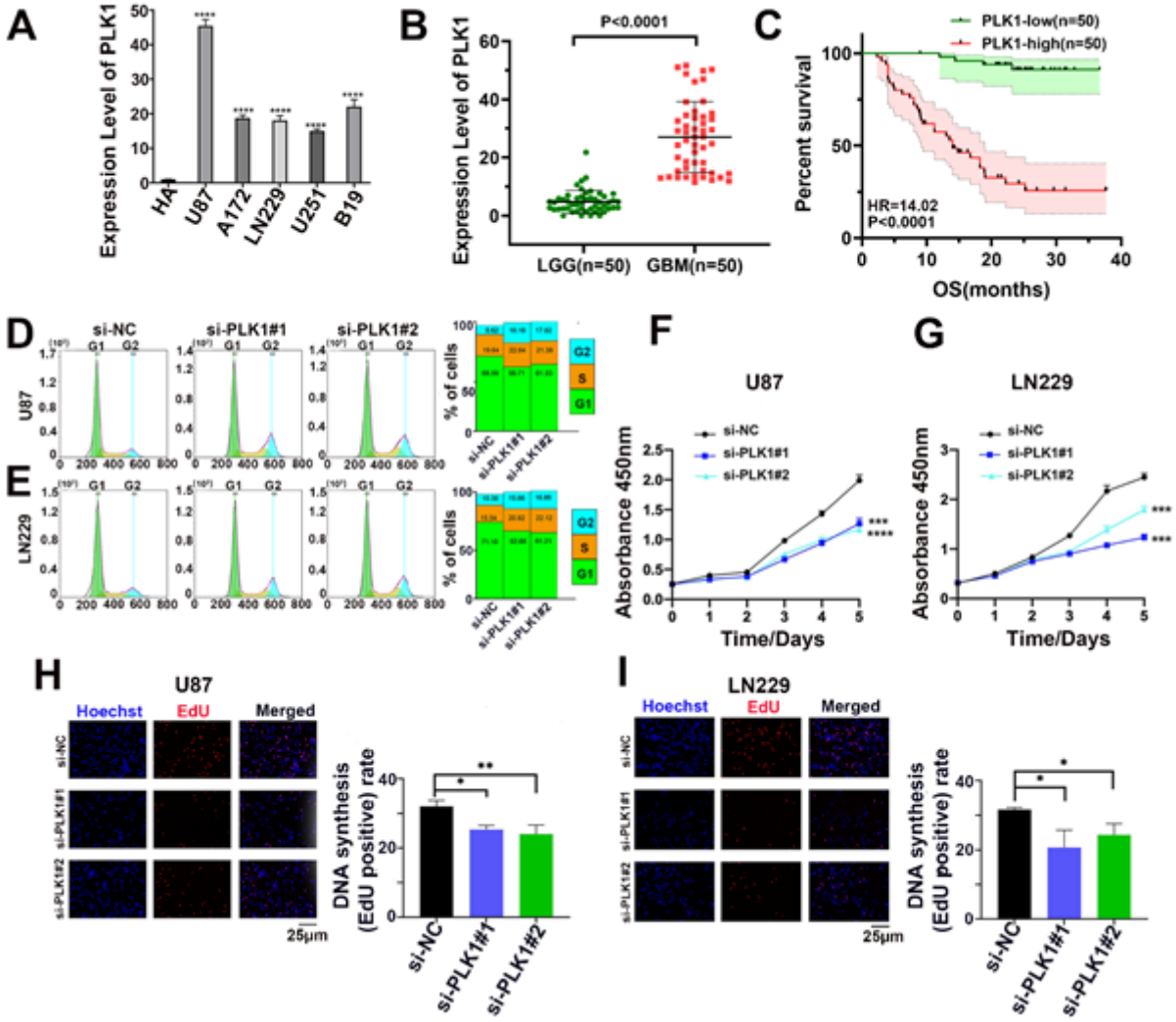

Figure 7

Experimental verification of PLK1 expression and phenotype in glioma.

(A). The expression levels of the PLK1 mRNA in NHA cell and five different glioma cell lines (U87, U251, LN229, A172 and B19) by qRT-PCR. (B). The correlation between PLK1 expression and grades in 100

glioma samples, including LGG (n=50) and GBM (n=50) samples; (C). Overall survival (OS) of different PLK1 expression level in 100 glioma samples. (D-E). Cell cycle distribution was evaluated using flow cytometry. (F-I). CCK8 and EdU assays were employed to measure cell viability proliferation.

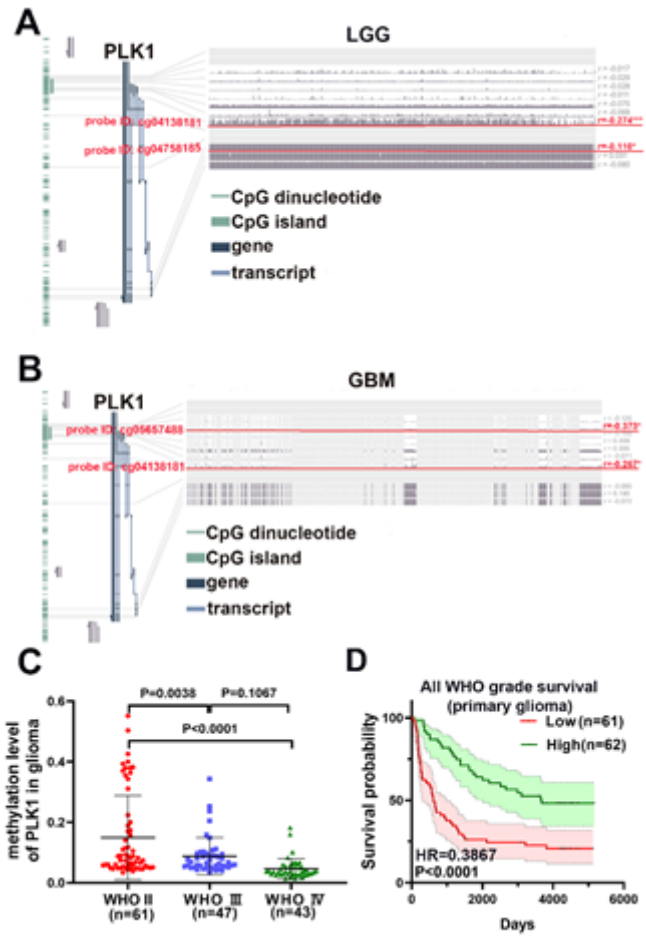

Figure 8

The DNA methylation levels of PLK1 in glioma.

PLK1 expression was negatively correlated with PLK1 DNA methylation in LGG (A) and GBM (B) across MEXPRESS. (C). Relationship between PLK1 methylation level and glioma WHO grades in CGGA datasets. (D). Relationship between PLK1 methylation level and prognosis in primary glioma in CGGA database.

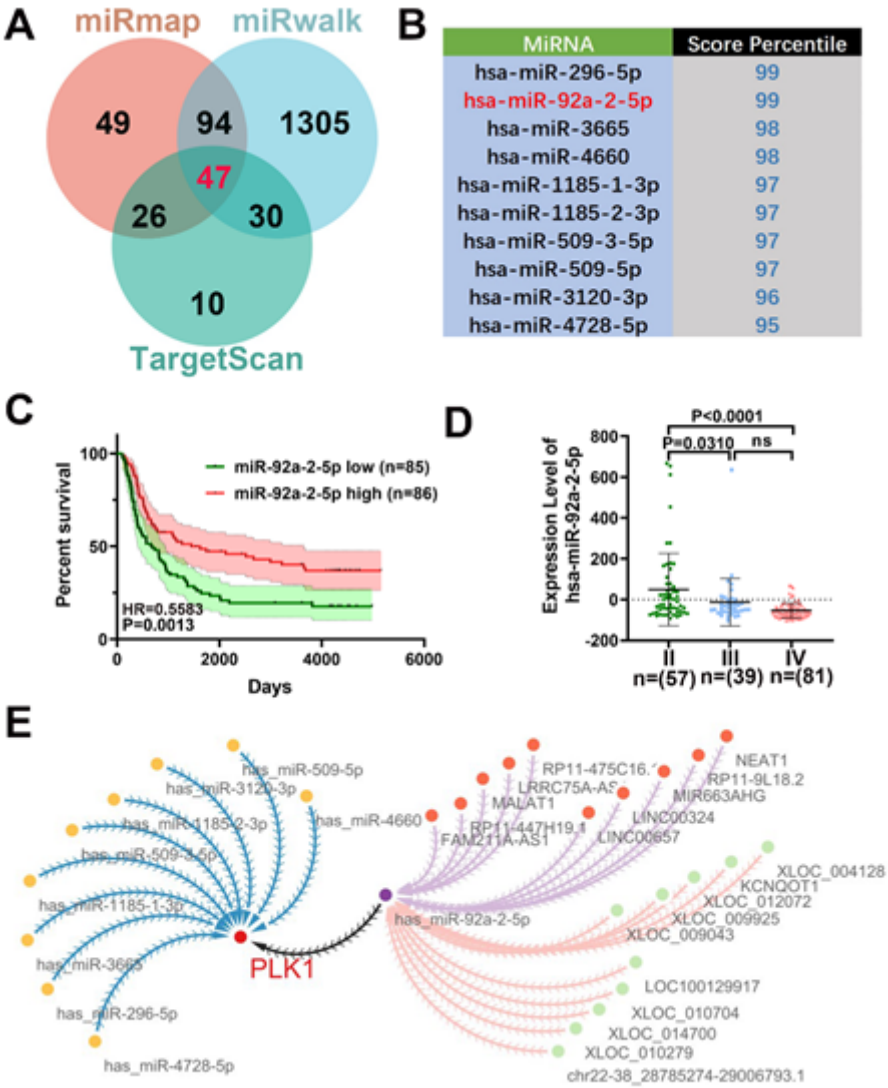

Figure 9

The lncRNA-miRNA-PLK1 regulatory network constructed.

(A). The upstream miRNAs of PLK1 were predicted by miRmap, miRwalk, and TargetScan databases and the intersection was taken (47 intersection miRNAs). (B). The top 10 miRNAs targeting PLK1 were displayed in TargetScan database. (C). The relationship between hsa-miR-92a-2-5p expression and prognosis in glioma patients from CGGA microRNA array dataset. (D) The relationship between hsa-miR-92a-2-5p expression and grades in glioma from CGGA microRNA array dataset. (E). The lncRNA-miRNA-PLK1 regulatory network was constructed by CytoScape.

Supplementary Files

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