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Research article

The prognostic values and immune characteristics of polo-like kinases (PLKs) family: A pan-cancer multi-omics analysis

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ABSTRACT

Background: In the realm of tumor-targeted therapeutics, Polo-like kinases (PLKs) are a significant group of protein kinases that were found recently as being related to tumors. However, the significance of PLKs in pan-cancer remains systematically studied.

Methods and materials: We integrated multi-omics data to comprehensively investigate the expression patterns of the PLK family across various cancer types. Subsequently, study examined the associations between tumor mutation burden (TMB), microsatellite instability (MSI), immune subtype classification, immune infiltration, tumor microenvironment scores, immune checkpoint gene expression, and the PLKs expression profiles within various tumor types. Furthermore, using our mRNA sequencing data (TRUCE01) and four bladder cancer (BLCA) cohorts (GSE111636, GSE176307, and IMvigor210), We examined the correlation between the expression level of PLK and immunotherapy effectiveness. Next, Gene set enrichment analysis (GSEA) was evaluated to find that potentially enriched PLK signaling pathways. Utilizing TIMER 2.0, we conducted an immune infiltration analysis underlying transcriptome expression, copy number variations (CNV), or somatic mutations of PLKs in BLCA. Finally, mRNA expression validation of PLK1/3/4 by real-time PCR within 10 paired BLCA tissues, protein expression verification through the Human Protein Atlas (HPA), and PLK4 in vitro cytological studies have been employed in BLCA.

Results: The expression of most of the PLK family members exhibits variation between cancerous tissues and adjacent normal tissues across various cancer species. Furthermore, the expression of PLKs demonstrates a significant association with immunotyping, infiltration of immune cell, tumor mutational burden (TMB), microsatellite instability (MSI), immunological checkpoint gene activity and therapeutic effectiveness in pan-tumor tissues. Additional investigation into the correlation between the PLK family and BLCA has revealed that the expression of the PLK genes holds substantial significance in the biological processes of BLCA. Furthermore, a notable association has been observed between the copy number variation, variant status, and the degree of certain immunological cell infiltration. Of note, the expression validation and in vitro phenotypic

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experiments have demonstrated that PLK4 has a significant function in promoting the BLCA cell proliferation, migration, and invasion.
Conclusion: Collectively, based on various databases, our results highlight the involvement of PLK gene family in the formation of different types of tumors and identify PLK-related genes that may be used for therapy.

Abbreviations

PLKs	Polo-like kinases
TMB	Tumor mutation burden
MSI	Microsatellite instability
TCGA	The Cancer Genome Atlas
PCR	Polymerase Chain Reaction
PBD	Polo-box domain
HPA	Human Protein Atlas
CNVs	Copy number variations
SNVs	Single nucleotide variations
OS	OverallSurvival
DFS	Disease Free Survival
DSS	Disease Support Survival
PFS	Progression Support Survival
TGCT	Testicular germ cell tumors; LGG, brain lower grade glioma
ACC	Adrenocortical Cancer
BLCA	Bladder Cancer
BRCA	Breast Cancer
CESC	Cervical Cancer
CHOL	Bile Duct Cancer
COAD	Colon Cancer
DLBC	Large B-cell Lymphoma
ESCA	Esophageal carcinoma
GBM	Glioblastoma
HNSC	Head and Neck Cancer
KICH	Kidney Chromophobe
KIRC	Kidney Clear Cell Carcinoma
KIRP	Kidney Papillary Cell Carcinoma
LAML	Acute Myeloid Leukemia
LGG	Lower Grade Glioma
LIHC	Liver Cancer
LUAD	Lung Adenocarcinoma
LUNG	Lung Cancer
LUSC	Lung Squamous Cell Carcinoma
MESO	Mesothelioma
OV	Ovarian Cancer
PAAD	Pancreatic Cancer
PCPG	Pheochromocytoma & Paraganglioma
PRAD	Prostate Cancer
READ	Rectal Cancer
SARC	Sarcoma
SKCM	Melanoma
STAD	Stomach Cancer
TGCT	Testicular Cancer
THCA	Thyroid Cancer
THYM	Thymoma
UCEC	Endometrioid Cancer
UCS	Uterine Carcinosarcoma
UVM	Ocular melanomas.

1. Introduction

Worldwide, cancer has been a public health problem for a long time and can be caused by many different factors [1]. The identification and study of key tumor genes have important significance in the occurrence, maintenance, and progression of cancer [2]. Large sample, high-throughput data will facilitate our study of key tumor genes. We may perform pan-cancer research in the multiomics literature because several public medical databases, such as the TCGA, supply us with multiomics information on various malignancies [3].

Tumor immunotherapy is an emerging topic in the field of tumor therapy [4]. Immunotherapy has gradually attracted attention because of its distinct benefits in avoiding tumor spread and recurrence owing to minimal adverse effects and excellent specificity [5]. Several methods are included in cancer immunotherapy, involving immune checkpoint inhibitors (ICIs) based on monoclonal antibodies (mAbs), therapeutic antibodies, tumor vaccines, therapeutic cells, small molecule inhibitors etc. [6]. In recent years, the use of novel immune checkpoint inhibitors such as Programmed Cell Death Ligand 1 (PD-L1) and Programmed Cell Death 1 (PD-1) has demonstrated considerable promise in the treatment of several cancer types [7,8]. The most prevalent cancer in the urinary tract is bladder carcinoma, which can be attributed to multiple genetic factors and its alterations [9]. The immunological milieu present in tumors is a prerequisite for immunotherapy and contributes to the cancer development. The C-terminal Polo-box domain (PBD) of Polo-like kinases (PLKs), a class of highly conserved serine/threonine protein kinases involved in various phases of eukaryotic mitosis and cytokinesis, attaches to PLK substrates, targets PLK, and modulates Plk action [10]. Currently, multiple research showed that aberrant expression of PLK family has a strong correlation between the initiating, spreading, and patient prognosis of malignancies [11–13]. Given this, PLK has become considered a biomarker and a potentially useful candidate for treatment [14,15]. Currently, several PLK inhibitors are already in clinical use and some of them are currently in clinical development, like BI 2536 [16]. However, the majority of prior research for the PLK family has been confined to specific types of cancer or individual members of the PLK family. Limited attention has been given to exploring the comprehensive relationship between the entire PLK family, consisting of five members, and pan-cancer, particularly in its correlations with immunological characteristics or immune responses.

In the present study, we performed in-depth bioinformatics analysis to investigate PLK family in pan-tumor, from a comprehensive multi-omics and multi-dataset standpoint. Meanwhile, the correlation between immunological subtype and infiltrated immune cells and PLK-expressed genes, MSI, TME, cancerous stem cell index, immune checkpoint, and immunotherapy efficacy was also examined. Moreover, study assessed the association between multiple signaling pathways and PLK expression patterns in human BLCA. Furthermore, we examined the relationship between alterations in the copy number variations of the PLK family gene and the degree of infiltration of immune cells. In addition, we conducted expression validation through the qRT-PCR method and the Human Protein Atlas (HPA) database. Following PLK4 knockdown, several in vitro cell functioning studies were carried out. The primary objectives of this study were to use multi-dimensional correlational analyses to clarify the biological functions of PLKs and provide a valuable resource for subsequent studies about the identification, treatment, and diagnosis of the PLK family in a variety of cancers.

2. Methods and materials

2.1. Acquisition of data

Normalized gene expression RNAseq data (namely, the HTSeq-FPKM value) and clinical information (such as age, gender, TCGA molecular typing, stage, and stem cell scores, etc.) was downloaded for 33 cancer types from UCSC Xena Browser (https://xenabrowser.net/) [17]. Furthermore, Genetic alteration data, including mutation and CNV information of bladder cancer has been retrieved from cBioPortal (http://www.cbioportal.org/). Using the relevant microarray platform files, GPL17586 and GPL24014, quantile normalization and annotation were carried out on the microarray expression data from two GEO immunotherapy databases, GSE111636 (n = 11) and GSE176307 (n = 89). Additionally, the IMvigor210 mRNA-sequencing dataset, comprising 298 MIBC individuals who received atezolizumab (a PD-L1 inhibitor), validated PLK family genes as possible indicators of immunotherapy effectiveness.

Furthermore, the mRNA transcriptome sequencing data and clinical data of 29 cases which comprised 15 tumor tissues at baseline and 15 paired tumor tissues following immunotherapy were obtained as a consequence of our continuous single-arm phase II clinical trial combining low-dose nab-paclitaxel with tislelizumab for muscle-invasive bladder tumors. The paired Wilcoxon test has been employed to predict the statistical significance of the differential expression, allowing us to examine variations in the expression of PLK. Individuals with pathologies determined by surgery (RC-PLND or maximal TURBT) who reported complete or partial responses (CR/PR) to immunotherapy were included in the study; patients with static or progressive disease (SD/PD) were considered non-responders.

2.2. Analysis of PLKs family expression and prognostic analysis in pan-cancer

First, to develop a block diagram that shows the expression levels of the 5 PLK family members (PLK 1–5), we used the transcription information collected from 33 TCGA tumors and deleted the normal facts. After that, a statistical study was conducted out to compare gene expression profiles of 18 common tumor types, including over 5 representative samples, with healthy and malignant samples. “Finally, the differential expression levels of PLK families were analyzed in 18 tumors (including BLCA, CHOL, KIRP, LUAD, BRCA, ESCA, COAD, PRAD, LUSC, HNSC, GBM, KICH, LIHC, THCA, KIRC, STAD, READ, and UCEC). Differences between adjacent regular and

cancerous tissues have been examined by the Wilcox test and further analysis of the correlation between PLK genes by the R package corrplot.

Afterwards, the Kaplan-Meier curves (K-M) were generated using the ideal cutoff that was established devoted the "surv_cutpoint" function from the "survival" R package to show variations in the survival of patients. Additionally, the probability proportion of PLK activity was determined for each TCGA cancer type in terms of Overall Survival (OS), Disease-Free Survival (DFS), Disease-Specific Survival (DSS), and Progression-Free Survival (PFS) using univariate cox regression analysis. To find the genes having independent prognostic significance, multivariate Cox regression analysis of PLK family genes in combination with clinical variables was further performed."

2.3. Correlation analysis of PLKs expression and immunological subtypes, tumor microenvironment and stemness indices

The relationship between PLK activity and the immunological subgroups of pan-cancer has been evaluated by utilizing the TCGA database. When the P value was less than 0.05, the differences were deemed to be statistically significant. Using the estimation algorithm, we obtained an immune score and a stroma score separately from all TCGA cancer sample. Then, the association between the immune/matrix scores and the PLK level of expression has been determined by the Spearman correlation analysis. Finally, we obtained a correlation between PLK expression and a stemness index based on DNA methylation (DNAss)/mRNA expression (RNAss) in the 33 TCGA tumors [18].

2.4. Correlation studies between the expression of PLKs and microsatellite instability (MSI), tumor mutational burden (TMB) and immunological checkpoint expression

In 33 TCGA cancers, the Spearman correlation analysis was used to find a relationship between PLK activity and TMB, MSI, or immune-mediated checkpoints (such as PD-L1, PD-1, and CTLA 4).

3. Timer

Tumor Immune Estimation Resource 2.0 (TIMER 2.0; http://timer.comp-genomics.org/) serve as a dependable tool offering systematic evaluations of various immunologic cell infiltrations. Therefore, we employed TIMER to examine the relationship between the gene expression of every member of the PLK family and the extent of infiltrated immune cells in multiple pan-carcinoma species.

3.1. Screening drugs using the expression of TRPVs

The CellMiner dataset can be utilized to select drugs that were either inefficient or efficient when the expression of PLK was altered through an association study between PLK expression and drug susceptibility. Using the z-score value, the treatment efficacy was calculated.

3.2. Explored the correlation between PLK family expression and cancer immunotherapy effectiveness

The predictive significance of PLK expression in the effect of immunotherapy was examined by downloading gene expression profiles, clinical data, and one of our sequencing datasets (TRUCE01) from 3 distinct cohorts (GSE111636, GSE176307, and IMvigor210). We also examined the differences between the two groups in our TRUCE01 pre- and post-therapy by employing a paired Wilcoxon analysi for PLK family genes. With a statistical threshold of $p < 0.05$ for difference, the data has been classified marked responded and non-responded.

3.3. Gene set enrichment analysis (GSEA)

The Molecular Signatures Database (MSigDB) of the Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets were used in GSEA to determine the biological roles and possible signaling paths of PLKs in bladder tumors.

3.4. Association analyses of immune subtype, clinicopathologic characteristics and tumor microenvironment and PLKs expression in bladder cancer

To investigate the relationship between the PLK level of expression and immunological subtypes and the clinicopathological features of BLCA such as age, gender, cancer level, tumor node metastasis (TNM) phase, and tumor subtype (non-papillary or papillary, we employed differential analysis utilizing the Wilcox or Kruskal test. Next, PLK expression of genes in BLCA has been associated with an immunological score, stromal score, estimated score, DNAss, RNAss, TMB, and MSI using the Spearman correlation test.

3.5. Analysis of infiltration of the immune cell in bladder tumors

Using the TIMER 2.0 dataset, we first investigated the linkage of PLKs in 6 key immune cell types (B cells, CD4 + T-cells, CD8 + T-cells, macrophages, neutrophils, and dendritic cells) in BLCA. Additionally, TIMER 2.0 was also used to examine the relationship

between the level of invading immune cells, copy number alterations, and bmp mutant status.

3.6. Extracting RNA, quantitative polymerase chain reaction (PCR), and analysis of the human protein atlas (HPA)

Using the E. Z.N.A.TM Hp total RNA Kit (OMEGA), total RNA was collected from ten paired BLCA cancers and nearby tissues. The

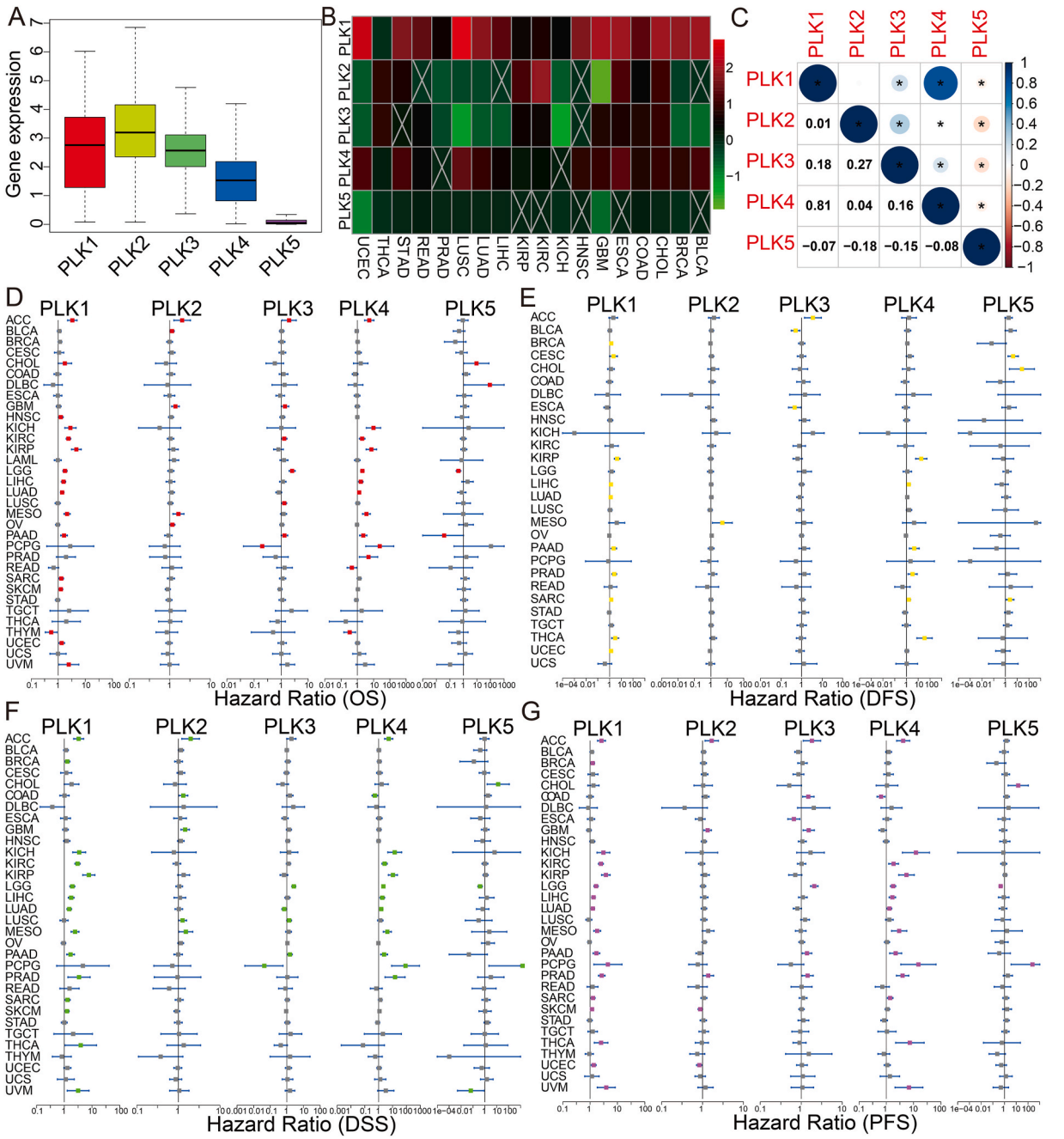

Fig. 1. Gene expression levels, correlation and clinic prognosis of the PLK family acorss 33 pan-cancer types from TCGA. **(A)** Expression of PLK family genes in various cancers. **(B)** Compared with expression in adjacent tissues, each PLK genes expression in different types of cancer based on TCGA data. In each small rectangle, red indicates significantly higher expression and green represents lower expression of the PLK family gene. **(C)** Correlation between PLK family genes. Blue dots represent positive correlation, while red dots represent negative correlation. The forest plots were calculated using univariate Cox regression for **(D)** OS (overall survival), **(E)** DFS (disease-free survival), **(F)** DSS (disease-specific survival), and **(G)** PFS (Progression-free Survival). Colors represent significance level of Univariate Cox analysis (red, yellow, green, and pink: $p < 0.05$; gray: non-significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Rockford, IL, USA) was then used to convert the extracted total RNA into cDNA. The relative expressions of PLK1, PLK3, and PLK4 mRNAs were ascertained by quantitative reverse-transcription PCR (qRT-PCR).

The PLK1 primer sequences were: forward, 5′-CACCAGCACGTCGTAGGATTC-3'; reverse, 5′- CCGTAGGTAGTATCGGGCCTC-3'. PLK3 primer: forward, 5′-AGCGCCTACGCTGTCAAAG-3'; reverse, 5′-CTCAAAGTGGTGCGAAAAACG-3'. PLK4 primer: forward, 5′-TTCTCGATACCTTCGTAGAGCTT-3'; reverse, 5′-CTGAGTGACATCGTTCCATTGT-3'. The control gene was GAPDH; the forward primer was 5′-CGGAGTCAACGGATTTGGTC-3′, and the reverse primer was 5′-TTCCCGTTCTCAGCCTTGAC-3'. The $2^{-\Delta\Delta CT}$ technique was used for analyzing the final results. Furthermore, these genes expression was further verified using the HPA database.

3.7. CCK-8, transwell migration and invasion assays

After transfection, 1.5 × 10ˆ3 BLCA cells were grown into plate with 96 wells and then cultivated for 120 h in 5% carbon dioxide (CO2) at 37 °C. A plate with 96 wells was used for the cell seeding. "To each well, 90 μl of new medium and 10 μl of CCK-8 solution (Boster Bio) were added. The optical density at 405 nm was measured after 4 h in the dark using a VersaMax Microplate Reader. For cell migration and invasion assays, 5 × 10ˆ4 cells were seeded with 200 μl of serum-free medium in the upper chamber (0.8 μm; Corning) and 700 μl complete medium in the lower chamber with (for invasion assays) or without (for migration assays) the Matrigel (Corning). The BLCA cells were incubated for 48 h before being fixed and stained with 0.1% crystal violet (Solarbio) and 4% para-formaldehyde (Sigma). Cell invasion and migration were measured with an Olympus microscope."

3.8. Statistical analysis

The R software program (version 4.0.2) and R were used to calculate the entire analysis. When comparing two groups, Wilcoxon rank sum test were performed, while Kruskal-Wallis tests were used for comparisons of three or more groups. Using Spearman's correlational coefficient analysis, a correlational evaluation has been performed. The log-ranked test was employed to ascertain statistical significance within each subgroup, and Kaplan-Meier curve analysis was utilized to evaluate survival between both low and high-expression cohorts. Each dataset from different platforms was separately compiled and analyzed due to the batch effect. Employing both univariate and multivariate Cox regression, the independent predictive effects of PLK genes for OS, DFS, DSS, and PFS in 33 tumor forms have been studied. In this study, statistical significance was defined as P-values less than 0.05. To provide strict control for any false discoveries, the False Discovery Rate (FDR) method was employed to alter each p-value across various comparisons.

4. Results

4.1. Differential co-expression analysis of PLK family members in pan-cancer

A comprehensive structure diagram of the present study has been found in Supplementary Fig. S1. Firstly, we analyzed the expression level of mRNA of the five-member genes within the PLK family across 33 types of cancer (Fig. 1A), and that of each member gene in the 33 cancer tissues separately and ranked them from high to low (Supplementary Fig. S2A), based on the TCGA database. The results found that in pan-cancerous samples, PLK2 had the highest expression compared to the other PLK family members, followed by PLK1, PLK3 and PLK4, whereas PLK5 exhibited the lowest expression. In specific cancer tissues, the cancer tissue types with the highest expression ranged from PLK1 to PLK5 were DLBC, KIRC, PAAD, LAML, and LGG.

Afterwards, we further analyzed PLK family genes expression in various tumors and matched para-tumor tissue, finding that the most of the PLK family genes were differentially expressed in a variety of cancers (Fig. 1B; Supplementary Fig. S2B). Afterwards, both the tumor group and the PLK4 group significant results showed a considerable increase in PLK1 expressed genes compared to the normal group. **Additional data 1** highlights these variations with the p-value and FDR-corrected results. Following that, the expression of PLK family genes in each type of tumor was then correlated by using the Spearman correlation analysis (**Additional data 2**). As seen in Fig. 1C, there were significantly positive or negative associations, including PLK1 and PLK4 (correlation coefficient (Cor) = 0.81), PLK2 and PLK3 (Cor = 0.50), PLK2 and PLK5 (Cor = −0.18).

4.2. Prognostic analysis of PLK family genes in pan-cancer

We examined the association between PLK activity and survival prognostic in 33 TCGA cancers to assess the influence of PLK family gene activity on prognosis. Initially, by setting the optimal cutoff determined by the "survival" R package, we performed KM analysis of survival to assess the survival difference of OS, DFS, DSS, and PFS across individuals with low and high PLK expression (Supplementary Table S1; Additional Data 3). In multiple cancers, nearly all PLK family members have a strong positive or negative correlation with OS, DFS, DSS, and PFS with considerable predictive conformity. Next, using univariate Cox regression, we also demonstrated how the expression of PLK family members was related to OS duration in a variety of tumor forms (Fig. 1D). The majority of PLK genes were analyzed with similar prognosis for DFS, DSS, or PFS by the same method (Fig. 1E–G). Interestingly, the statistically significant prognosis for PLK genes in Fig. 1D–G had mostly corresponding same-direction K-M curves. As can be seen, high expression of PLK1 or PLK4 was a high-risk factor for those who had various tumors types were and is linked to an unfavorable outcome.

Afterwards, the PLK family genes and prevalent clinical features (age, sex, and tumor stage) were subjected to multivariate Cox

regression analyses to determine that any of the PLK genes independently predicted OS, DFS, DSS, and PFS (Tables 1–4; Supplementary Tables S2–5). Of them, with three or more distinct cancer types, PLK1 and PLK4 can be negative predicting factors for OS, DFS, DSS, and PFS (HR > 1, the P value < 0.05), except for the KICH cancer in DFS (HR < 1, P-value<0.05). Notably, PLK2 independently predicted poorer outcomes for OS, DSS, and PFS (instead of DFS) in ACC cancer (HR > 1, P-value<0.05), while PLK3 predicted favorable results for PFS in BLCA, CHOL, and ESCA (HR < 1, P-value<0.05).

4.3. Correlation of PLK genes expression and clinicopathologic parameters

Usually, patients with high tumor stages have a poor prognosis. Herein, we explored the correlations between each PLK family gene and tumor stage by the Kruskal-Wallis test (Supplementary Fig. S3), as well as between each PLK family gene and first course treatment outcome through the Wilcoxon test (Supplementary Fig. S4). Attractively, the PLK1 or PLK4 expression level is gradually significantly elevated with the progress of tumor stages, in tumors of ACC, BRCA, KIRP, etc. The PLK1 or PLK4 expression level also increased with the development of the TGCT stage of malignancy. PLK2 level of expression also tended to decrease with increasing HNSC cancer stag (Supplementary Figs. S3A–E).

Conversely, we investigated the relationship between the results of the initial session of therapy and PLK expression of gene (Supplementary Figs. S4A–E). Notably, PLK1 or PLK4 expression was significantly higher in the SD/PD group compared to the CR/PR group following first-course therapy for several tumor types. Compared to the CR/PR group, PLK2 and PLK3 activity was increased in PRAD tumors; TRPV2 in UCEC, TRPV2 in LUAD, and PLK5 in ESCA and LGG were all lower in the SD/PD group.

4.4. Correlation analyses of the PLK expression with immunization typing, cancer stemness scores, or tumor microenvironment in pan-cancer

This study examined the relationship between six different subtypes of immune infiltrates found in the research by David et al. and the expression of PLK genes in pan-cancer tissue. These subtypes included C1 (healing of wounds), C2 (dominance of IFN-γ), C3 (inflammation), C4 (depletion of lymphocytes), C5 (immune quiescence), and C6 (dominance of TGF-β) [19] (Fig. 2A). In pan-cancerous tissues, PLK2 expression levels showed the greatest in C6 and the smallest in C5. The overall trend of PLK3 in each subtype was basically the same as that of PLK2, except PLK3 overall expression is significantly lower than PLK2. The trend of PLK1 expression in different immunophenotypes was similar to that of PLK4; the gene PLK1 gene expression or C2 subtype had the highest PLK4 level. And, the expression of PLK5, compared to the other PLK family members, exhibited generally low expression within each subtype and was the highest in the C5 subtype. Meanwhile, we found a significant correlation between PLK3 or PLK4 activity and the primary immune effector cell infiltration in pan-cancerous carcinoma tissues after examining the association between PLK expression

Table 1
The brief results of multivariate Cox regression analysis of each PLK member and common clinical traits (i.e. age, gender, and cancer staging) for OS acorss different tumor types.

CancerType	PLK1		PLK2		PLK3		PLK4		PLK5	
	HR	pvalue	HR	pvalue	HR	pvalue	HR	pvalue	HR	pvalue
ACC	3.49	0	1.85	0.014	1.69	0.118	3.47	0.002	0.89	0.797
BLCA	1.08	0.35	1.14	0.028	1	0.99	0.93	0.577	0.43	0.102
BRCA	1.23	0.01	1.02	0.843	1.2	0.155	1.12	0.35	0.17	0.072
CESC	1	0.996	1.13	0.198	1.11	0.439	1.13	0.551	0.74	0.54
CHOL	1.74	0.07	0.83	0.597	0.56	0.136	1.6	0.385	16.11	0.022
COAD	0.96	0.77	1.01	0.901	1.04	0.833	0.88	0.534	1.42	0.233
DLBC	0.77	0.506	0.75	0.732	1.07	0.916	0.78	0.627	1430.2	0.021
ESCA	1.08	0.636	0.95	0.761	0.81	0.262	1.17	0.483	1.96	0.314
HNSC	1.25	0.027	1.1	0.19	1.14	0.229	1.03	0.81	0.76	0.657
KICH	3.8	0	0.83	0.747	1.88	0.228	18.36	0.001	>1000	0.128
KIRC	1.95	0	1.11	0.303	1.41	0.009	1.82	0.005	1.17	0.528
KIRP	3.36	0	1.23	0.223	1.23	0.405	4.29	0.002	1.03	0.933
LIHC	1.47	0	0.98	0.885	1.13	0.41	1.41	0.048	1.83	0.214
LUAD	1.39	0	1.1	0.235	0.89	0.352	1.35	0.005	0.91	0.807
LUSC	0.98	0.829	1.07	0.359	1.28	0.012	0.99	0.925	1.02	0.974
MESO	2.09	0	1.64	0.002	1.24	0.091	3.51	0	0.86	0.93
OV	0.99	0.909	1.14	0.072	1.08	0.376	1	0.966	1.72	0.39
PAAD	1.52	0.005	1.08	0.513	1.26	0.104	2.01	0.008	0.05	0.062
READ	0.85	0.622	1.52	0.208	1.37	0.52	0.57	0.2	0.36	0.502
SKCM	1.33	0.002	0.99	0.861	0.88	0.173	1.1	0.443	0.7	0.571
STAD	0.91	0.362	0.91	0.319	0.99	0.949	0.77	0.077	1.21	0.531
TGCT	>1000	0.944	0.92	0.949	0.67	0.767	>1000	0.98	0.39	0.572
THCA	0.95	0.943	1.71	0.12	1.14	0.707	0.25	0.263	4.32	0.814
THYM	0.68	0.206	0.58	0.08	0.65	0.653	0.44	0.069	0.52	0.425
UCEC	1.13	0.319	1.05	0.619	0.98	0.919	1	0.984	0.76	0.645
UCS	0.98	0.955	0.88	0.471	1.13	0.755	0.91	0.846	2.68	0.168
UVM	2.39	0.035	1.24	0.487	1.8	0.071	2.66	0.15	0.08	0.051

Table 2
The brief results of multivariate Cox regression analysis of each PLK member and common clinical traits (i.e. age, gender, and cancer staging) for DFS acorss different tumor types.

CancerType	PLK1		PLK2		PLK3		PLK4		PLK5	
	HR	pvalue	HR	pvalue	HR	pvalue	HR	pvalue	HR	pvalue
ACC	1.56	0.379	1.53	0.257	3.38	0.015	0.86	0.861	1.87	0.223
BLCA	1.07	0.713	1.08	0.619	0.51	0.006	1.27	0.408	3.38	0.035
BRCA	1.4	0.002	0.94	0.58	1.07	0.722	1.16	0.381	0.05	0.061
CESC	2.29	0.03	1.1	0.535	1.01	0.97	1.8	0.095	5.6	0.001
CHOL	1.47	0.227	1.04	0.932	1.21	0.702	1.98	0.297	27.9	0.023
COAD	1.26	0.498	1.11	0.644	1.06	0.873	1.02	0.967	0.41	0.492
DLBC	0	1	3.01	1	>1000	1	>1000	1	>1000	1
ESCA	0.54	0.056	0.85	0.524	0.48	0.077	0.81	0.568	2.06	0.311
HNSC	1.17	0.536	1.35	0.178	1.29	0.405	1.07	0.817	0.02	0.314
KICH	0	0.994	1.08	0.932	>1000	0.966	0	0.035	0	0.408
KIRC	1.12	0.86	1.02	0.961	0.84	0.676	0.94	0.929	0.44	0.67
KIRP	3.12	0.001	0.94	0.709	0.73	0.249	8.79	0	0.54	0.666
LIHC	1.19	0.042	0.95	0.659	0.98	0.901	1.36	0.064	0.45	0.229
LUAD	1.26	0.036	1.05	0.7	0.82	0.249	1.21	0.223	1.13	0.775
LUSC	1.06	0.751	0.94	0.672	0.9	0.599	1.44	0.189	1.02	0.986
MESO	11.41	0.105	16.35	0.144	1.38	0.502	11.56	0.144	>1000	0.402
OV	0.93	0.527	1.12	0.206	1.02	0.835	0.89	0.444	0.45	0.445
PAAD	2.19	0.017	1.27	0.343	1.13	0.714	3.28	0.026	0.29	0.565
READ	0.78	0.722	0.37	0.271	0.83	0.854	0.18	0.086	2.41	0.711
STAD	0.92	0.648	1.11	0.543	1.16	0.604	0.75	0.264	1.67	0.192
TGCT	1.13	0.742	1.35	0.222	1.03	0.936	0.48	0.24	3.62	0.074
THCA	3.66	0.001	1.3	0.259	0.84	0.533	55.37	0	0.53	0.797
UCEC	1.27	0.108	0.91	0.401	0.83	0.401	1.06	0.744	0.77	0.705
UCS	0.27	0.121	0.91	0.767	1.27	0.762	1.01	0.996	0.57	0.728

Table 3
The brief results of multivariate Cox regression analysis of each PLK member and common clinical traits (i.e. age, gender, and cancer staging) for DSS acorss different tumor types.

CancerType	PLK1		PLK2		PLK3		PLK4		PLK5	
	HR	pvalue	HR	pvalue	HR	pvalue	HR	pvalue	HR	pvalue
ACC	3.53	0	1.84	0.018	1.68	0.142	3.43	0.002	0.89	0.804
BLCA	1.13	0.215	1.14	0.072	0.95	0.632	1.06	0.69	0.39	0.159
BRCA	1.34	0.007	1.04	0.746	1.27	0.168	1.25	0.178	0.09	0.11
CESC	1.08	0.734	1.16	0.176	0.97	0.873	1.1	0.678	0.92	0.865
CHOL	1.84	0.063	0.83	0.622	0.55	0.147	1.68	0.378	24.47	0.011
COAD	1.02	0.941	1.39	0.013	1.18	0.438	0.72	0.171	1.73	0.068
DLBC	0.63	0.371	0.19	0.34	2.95	0.393	0.8	0.726	0	0.314
ESCA	1.3	0.195	1.07	0.714	0.65	0.049	1.66	0.059	1.24	0.823
HNSC	1.2	0.17	1.22	0.032	1.16	0.276	0.97	0.812	0.5	0.414
KICH	92.24	0.043	1.28	0.695	2.46	0.129	>1000	0.094	>1000	0.07
KIRC	2.17	0	0.95	0.68	1.57	0.011	2.14	0.003	1.26	0.468
KIRP	3.94	0	1.25	0.278	0.98	0.96	7.13	0.001	1.25	0.521
LIHC	1.58	0.001	0.95	0.779	1.01	0.961	1.5	0.071	1.45	0.615
LUAD	1.45	0	1.02	0.885	0.73	0.054	1.52	0.003	0.83	0.771
LUSC	0.92	0.649	1.29	0.017	1.37	0.042	1.03	0.896	0.26	0.312
MESO	2.48	0	1.53	0.035	1.28	0.221	4.6	0	2.15	0.665
OV	0.94	0.503	1.12	0.166	1.09	0.343	0.97	0.792	2.09	0.257
PAAD	1.58	0.006	1.16	0.28	1.42	0.035	2.14	0.01	0.05	0.1
READ	1.36	0.508	0.82	0.629	0.62	0.466	0.68	0.54	0.93	0.953
SKCM	1.36	0.002	1.01	0.909	0.88	0.198	1.18	0.229	0.6	0.477
STAD	0.97	0.803	1	0.972	0.98	0.899	0.75	0.116	1.57	0.124
TGCT	>1000	0.956	0.96	0.975	0.65	0.79	>1000	0.984	0.43	0.645
THCA	3.01	0.269	1.96	0.174	0.54	0.174	0.05	0.124	16.82	0.512
THYM	1.17	0.726	0.25	0.023	1.87	0.663	0.83	0.793	0	0.031
UCEC	1	0.992	1.07	0.497	1	0.992	1.03	0.881	0.92	0.905
UCS	1.09	0.813	0.83	0.296	1.27	0.517	0.88	0.797	3.13	0.12
UVM	2.93	0.014	1.33	0.37	1.63	0.152	2.86	0.132	0.06	0.042

of genes and immune cell infiltration (Fig. 2B).

Furthermore, we studied the relationship of PLK with immunologic and stromal scores in pan-cancer tissues by employing the R package estimate (Fig. 2C–D). The findings demonstrated a strong positive association between immune or stromal scores and PLK2/PLK3 expression in multiple types of cancer; whereas, a remarkably negative correlation for PLK1/PLK4/PLK5 with immune or stromal

Table 4
The brief results of multivariate Cox regression analysis of each PLK member and common clinical traits (i.e. age, gender, and cancer staging) for PFS acorss different tumor types.

CancerType	PLK1		PLK2		PLK3		PLK4		PLK5	
	HR	pvalue	HR	pvalue	HR	pvalue	HR	pvalue	HR	pvalue
ACC	2.07	0	1.61	0.019	1.77	0.034	2.38	0.01	1.23	0.492
BLCA	1.13	0.12	1.04	0.494	0.82	0.036	1.24	0.078	0.89	0.796
BRCA	1.26	0.005	1.06	0.492	1.18	0.234	1.14	0.294	0.17	0.078
CESC	1.25	0.305	1.14	0.152	0.89	0.435	1.18	0.409	1.23	0.576
CHOL	1.32	0.273	1.13	0.674	0.48	0.035	1.04	0.936	26.92	0.005
COAD	0.97	0.815	1.21	0.049	1.27	0.134	0.78	0.148	1.38	0.222
DLBC	1	0.99	0.14	0.022	1.91	0.3	1.24	0.603	1.87	0.863
ESCA	1.15	0.367	0.88	0.373	0.64	0.011	1.28	0.193	1.32	0.64
HNSC	1.19	0.101	1.13	0.104	1.1	0.4	0.98	0.876	0.95	0.924
KICH	3.41	0.001	1.15	0.74	1.92	0.069	18.81	0.001	73.11	0.381
KIRC	1.66	0	0.96	0.702	1.08	0.575	1.36	0.168	0.98	0.957
KIRP	2.08	0.001	1.08	0.578	1.1	0.66	2.52	0.035	1.16	0.511
LIHC	1.18	0.04	1.03	0.767	1.08	0.561	1.39	0.028	0.82	0.718
LUAD	1.23	0.004	1.07	0.36	0.83	0.117	1.31	0.009	0.77	0.501
LUSC	0.87	0.283	1.13	0.153	1.26	0.054	1.16	0.386	0.65	0.579
MESO	1.85	0	1.27	0.179	0.99	0.959	3.6	0	1.27	0.883
OV	0.96	0.552	1.11	0.092	1.06	0.481	1.08	0.445	0.61	0.452
PAAD	1.56	0.002	0.96	0.717	1.23	0.139	1.98	0.011	0.39	0.412
READ	1.04	0.894	0.83	0.514	0.98	0.945	0.54	0.074	0.76	0.753
SKCM	1.27	0.002	0.98	0.687	0.94	0.403	1.21	0.063	1.02	0.967
STAD	0.96	0.718	1.07	0.485	1.08	0.623	0.82	0.183	1.38	0.228
TGCT	1.21	0.581	1.32	0.234	0.91	0.795	0.78	0.667	1.66	0.408
THCA	2.78	0.001	0.99	0.965	0.87	0.477	8.21	0.001	0.71	0.864
THYM	0.91	0.639	0.75	0.194	1.77	0.404	0.79	0.375	0.26	0.099
UCEC	1.2	0.087	0.87	0.082	0.94	0.678	1.08	0.563	0.67	0.429
UCS	1.2	0.532	0.81	0.171	1.24	0.543	1.04	0.934	2.45	0.211
UVM	3.54	0.001	1.46	0.119	1.14	0.685	6.2	0.002	0.62	0.56

scores. By the final step, tumor stemness scores in pan-cancer studies were compared to the PLK family genes using tumor stemness scores based on expression of mRNA (RNAss) and methylation of DNA pattern (DNAss) [18]. (Fig. 2E–F). Remarkably, the majority of tumors exhibit a negative association between the RNA stem cell index level and the PLK2/3 genes. The expression of PLK1/4 genes and stemcell scores derived from RNA or DNA sequencing exhibit a slightly significant positive correlation across multiple cancerious forms. The P-value and FDR-corrected values are included in Additional Data 4 and Additional Data 5, respectively, and with the detailed Spearman correlation data between PLK gene expression genes and immunological, stromal, RNAss, or DNAss.

4.5. Correlation of PLKs expression with TMB, MSI and immune checkpoint genes in pan-carcinoma

Immune checkpoint inhibitors (ICIs) were found to be well-predicted by TMB, MSI, and immune checkpoints-related genes. Hence, we assessed the latent correlations of PLK genes with these common ICIs indicators in 33 distinct types of cancer, comprising TMB, MSI, and ICI genes (Fig. 2G–I; Supplementary Figs. S5–7). PLK1/4 expression was strongly positively related to TMB in most tumor forms, excluding THYM, as illustrated in Fig. 2G and Supplementary Figs. S5A–E. Moreover, PLK2/3 expression with TMB having the strongest negative correlation in several types of cancer (including, BRCA and LIHC, etc). Similarly, we identified the activity of the PLK family genes was similarly linked to MSI in multiple cancer, for example, negative association across MSI and PLK2/3/4 expression in DLBC cancer. Interestingly, the expression of PLK1 revealed a substantial correlated positively with the MSI score in multiple types of cancer (Fig. 2H; Supplementary Figs. S6A–E). The P-value and FDR-adjusted correlations between the expression of PLK and TMB or MSI are displayed in **Additional Data 6**.

Next, we explored the relationship between each PLK family member and 33 different forms of cancer share 47 similar ICI genes (Supplementary Figs. S7A–E). Then, the three classic immune checkpoints (PD-1, PD-L1, and CTLA-4) have been further displayed in Fig. 2I. The findings indicated that PLK3 expression has been considerably positively correlated with the level of the three immunological checkpoints in a significant proportion of malignancies. In almost all the cancer types, except for TGCT and MHYM tumors, Immune checkpoint gene activity was favorably linked with PLK1/2/4 member expression. Notably, across a variety of cancer types, PLK5 expression was inversely correlated with those immune checkpoint genes. In **Additional data 7**, the results are also presented with P-value and FDR-corrected values.

4.6. Examination of PLK genes for drug sensitivity

In our study, using the CellMiner database, we examined the Spearman correlation with FDR correction between the expression of PLK genes and sensitivity to drugs. The results showed a significant association between PLK expression and the efficacy of several chemotherapeutic drugs ($p < 0.05$, Supplementary Table S6). As shown in Fig. 3A ($p < 0.01$), the PLK2 expression has been negatively

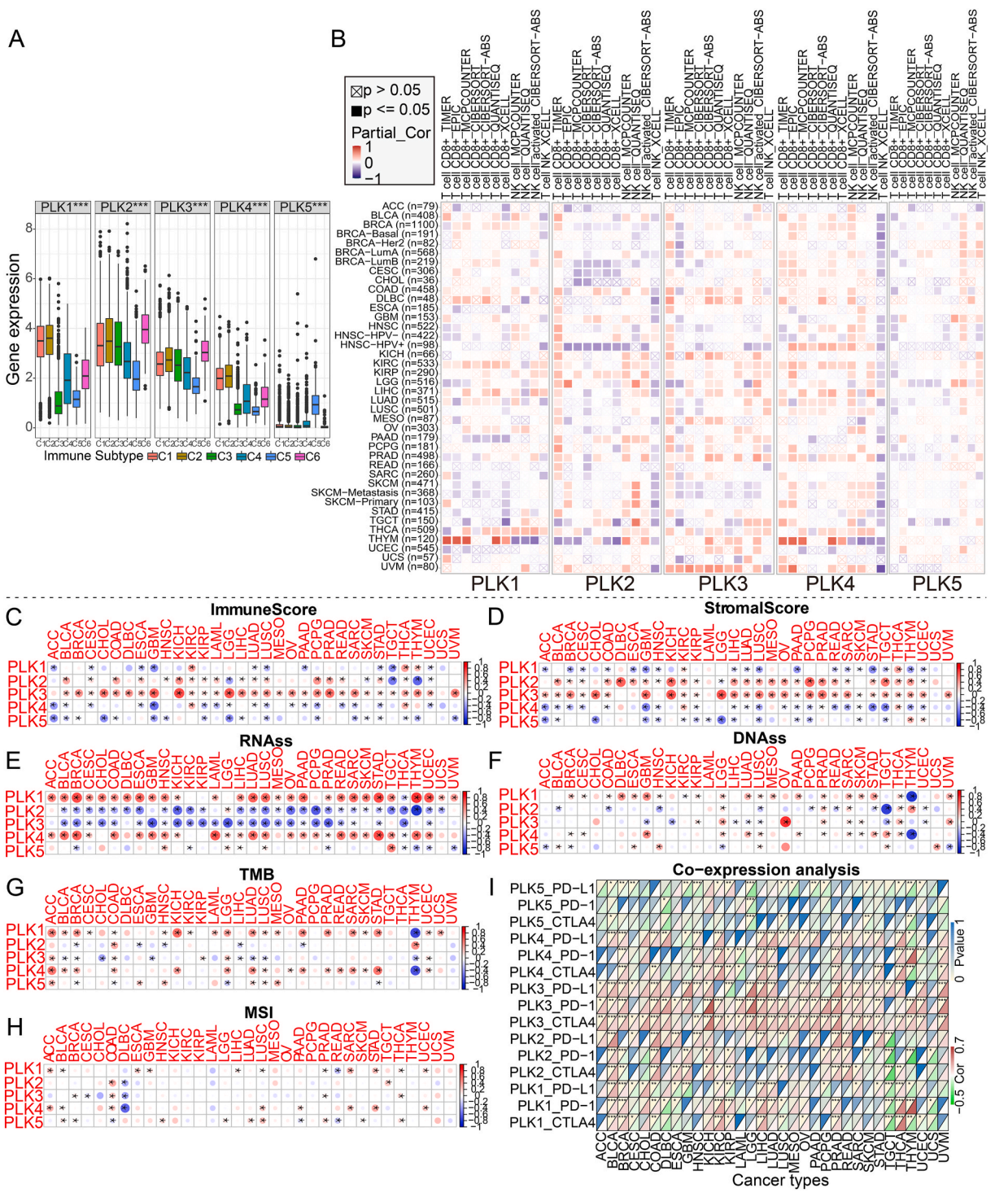

Fig. 2. Relation between PLKs expression levels and several immunological indicators in various tumors in TCGA database. **(A)** The relationship of expression of PLKs and immune subtypes based on TCGA database. C1 (wound healing), C2 (IFN-g dominant), C3 (inflammatory), C4 (lymphocyte depleted) and C6 (TGF-b dominant). **(B)** Several algorithms were conducted to analyze the potential correlation of PLKs expression level and the infiltration level of $CD8^+$ T cell, NK cell and NK-T cell. **(C–F)** Correlation between PLKs expression and immune score, stromal score, RNAss score or DNAss score. **(G)** Correlations between the PLKs and TMB. **(H)** Correlations between the PLKs and MSI. **(I)** Correlation between the expression of different PLK and three typical immune checkpoint genes (i.e., PD-L1, PD-1, and CTLA4).

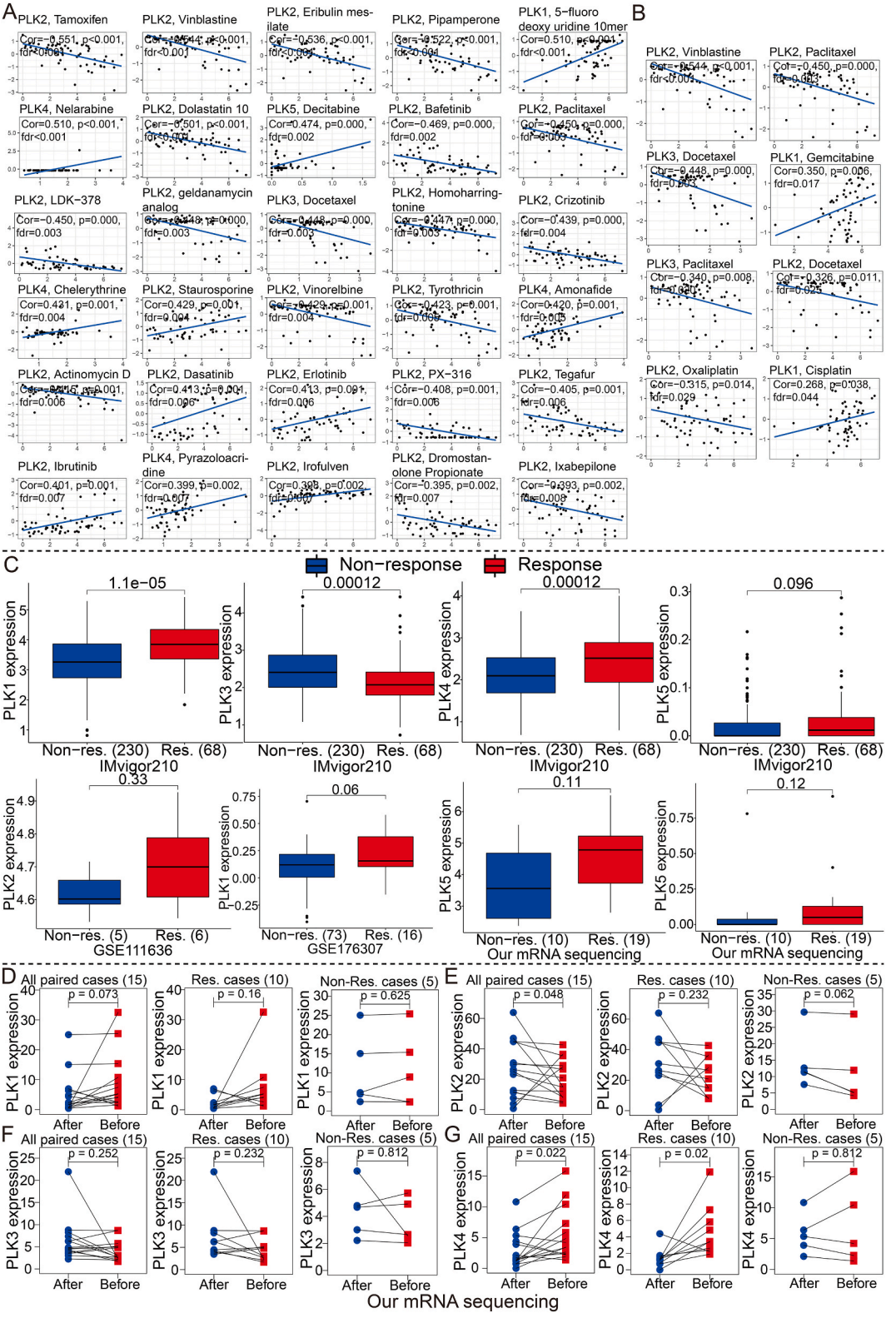

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Fig. 3. Relationship between PLK genes expression levels and small molecular chemotherapy drug sensitivity, and the efficacy of immunotherapy. **(A)** Top 30 drugs significantly associated with PLK family genes ($P < 0.01$) were obtained. **(B)** The significant correlation between commonly used chemotherapeutic drugs for cancer and the expression of PLK genes ($P < 0.05$). **(C)** Differential expression analysis was performed based on several immunotherapy cohort (including IMvigor210, GSE111636, GSE176307 and our own mRNA sequencing (TRUCE-01)). **(D–G)** The differences in PLKs expression before and after immunotherapy were compared by paired wilcox test in our TRUCE-01 data. FDR, false discovery rate.

correlated with the IC50 of 'Tamoxifen', 'Vinblastine', 'Eribulin mesilate', and 'Pipamperone', etc, this indicates these drugs were worked better in those with elevated ANXA2 expression levels. Additionally, PLK4 expression was found to have a positive correlation with the IC50 of Nelarabine, celerythrine, and amonafide. This indicates that individuals with elevated PLK4 expression should refrain from using these drugs. Since increased expression of PLK4 improves the drugs resistance. Besides that, we examined the relationship between PLK family member expression and the sensitivity of widely used chemotherapeutic agents for bladder cancer (Fig. 3B, $p < 0.05$). We found that there is a favorable correlation between PLK1 expression and the drug IC50 of cisplatin and gemcitabine. The IC50 of "Docetaxel", "Paclitaxel", or "Vinblastine" was adversely linked with other PLK family members (i.e., PLK2/3).

4.7. Correlation between PLK expression and the efficacy of immunotherapy"

Nowadays, immunotherapy plays a very important role in cancer treatment, so it is important to evaluate the association between the expression of gene and immunotherapy effect. In the four BLCA databases (i.e., TRUCE01, GSE116307, IMvigor210, and GSE111636), we studied the relationship between PLK expression and immunotherapy response. The objective responses of the Imvigor210 and GSE176307 groups to anti-PD-1/PD-L1 therapy were shown to be significantly correlated with PLK1/PLK4 expression level (Fig. 3C). The PLK2/PLK5 outcomes in Imvigor210, GSE111636, and our own TRUCE-01 group are similar (Fig. 3C). Conversely, we found that PLK3 expression had a positive relationship with cancer immunotherapy resistance (anti-PD-1/PD-L1) in the Imvigor210 (Fig. 3C). Overall, our findings suggested that the expression of PLK may be useful in predicting the response to anti-PD-1/PD-L1 immunotherapy." In addition, the P-value and FDR from these variations in the expression of PLK family gene between response and non-respond cohorts after PD-L1/PD-1 immunotherapy were shown in **Additional Data 8**.

In addition, we examined the potential processes underlying PLK expression of genes and the immunotherapy reaction. Our genetic sequence analysis revealed bladder cancer patients responding to tislelizumab with nab-paclitaxel therapy, the PLK1/PLK4 expression level considerably lowered after therapy; however, in non-responding instances, the increased level of PLK2 expression (Fig. 3D–G). Therefore, the molecular process causing such responses and the expression of PLK-family genes appeared to be strongly correlated with our findings. In the subsequent work, we carry out a more thorough analysis of this part. The Wilcox paired test was utilized to compare PLK gene expression changes after BLCA immunotherapy vs previously, as evidenced by the P-value and FDR values found in **Additional Data 9**.

4.8. The biological function of PLK in bladder cancer

We used the GSEA method to conduct KEGG pathway enrichment analyses to explore the significance of PLK family genes in the physiology of bladder tumors (Supplementary Figs. S8A–E; Supplementary Table S7). According to the results, upregulation of PLK1 may have a positive impact on the control of autophagy, the cell cycle, DNA replication, the pathway of JAK-STAT signaling, NK cell-mediated cytotoxicity, cytokine receptor interaction, antigen therapy, and presentation. Conversely, PLK2 overexpression favorably controls cytokine receptor interactions, NOD-like receptor signaling, NK cell-mediated cytotoxic effects, Leishmania infection, allograft rejection, chemokine signaling, JAK-STAT signaling, antigen processing and presentation, and T cell receptor signaling, etc. Upregulated PLK3 group were mainly enriched in pantothenic acid and coenzyme A biosynthesis, and other glycine degradation. And, downregulated PLK3 was primarily enriched in neuroactive ligand receptor interactions, systemic lupus erythematosus, etc. Finally, upregulation of PLK4 or PLK5 were mainly enriched in olfactory conduction, taste conduction, and neuroactive ligand receptor interactions, etc.

4.9. The relationship between PLK expression and immunotyping, clinical traits in bladder cancer

The results presented in Supplementary Fig. S9 indicate that the C6 subtype had a significant level of PLK3/PLK2 expression of genes, whereas the C2 subtype displayed higher PLK1/PLK2 expression levels compared with the other subtypes. Furthermore, PLK5 was less expressed in all immune phenotypes compared to other PLK family members, and PLK1 and PLK4 expression patterns were remarkably consistent across all immunological subtypes. The levels of expression of each PLK gene were subsequently determined to be significantly unrelated to age, sex, phase, metastasis of lymph nodes, and distant metastasis. Notably, PLK1/2/4 expression was enhanced in tissues with higher-grade bladder tumors, while PLK2 expression was elevated in tissues classified as late T.

4.10. Correlation studies of PLK genes expression with stemcell index, and immunological features in bladder cancer

As Supplementary Fig. S10 shows us, PLK1/4/5 were positively correlated with RNAss or DNAss, whereas PLK2, PLK3 and PLK4 were negatively correlated. PLK4 and PLK5 showed an unfavorable association with the three fractional scores of TME, but PLK2/3 showed a positive correlation. Through examination of the relationships among TMB, MSI, and PLK expression in bladder cancer, we

identified that PLK1/4 expression exhibits notable positive associations, albeit to varying degrees. The increased levels of PLK1, 2, 3, and 4 revealed a positive association with CD8 + T cells, however only the enhanced expression of PLK5 exhibited an adverse association (Supplementary Fig. S11). Based on our findings, immune cell infiltration within the tumor microenvironment and the survival of patients may be impacted by PLK family gene regulation.

4.11. *Copy number variations (CNVs), somatic variations of PLK family genes in bladder cancer*

The cBioPortal program was utilized to assess the variants, variation in copy number (CNVs), and single nucleotide variants (SNVs) of the PLKs family in bladder tumor. A Variant allele and frequency of PLKs family was estimated to be 13.73% in bladder cancer cohort contained 408 samples (Fig. 4A). The percentages of PLK family members with tumors who had altered genes ranged from 1 to 4%, as seen in Fig. 4B. The results of Kaplan–Meier curve and log-rank test showed significant statistical difference in overall and survival free from disease in individuals without or with have mutations in PLK genes (Fig. 4C and D). Afterwards, further details regarding the mutations of each member of the PLK family can be seen in Supplementary Figs. S12A–E, respectively. Additionally, we examined how each of their specific mutations affected the prospects of BLCA in comparison to those without mutations (Supplementary Fig. S12F-N). PLK3/4 mutation-carrying BLCA sufferers fare better than those with non-missense variants.

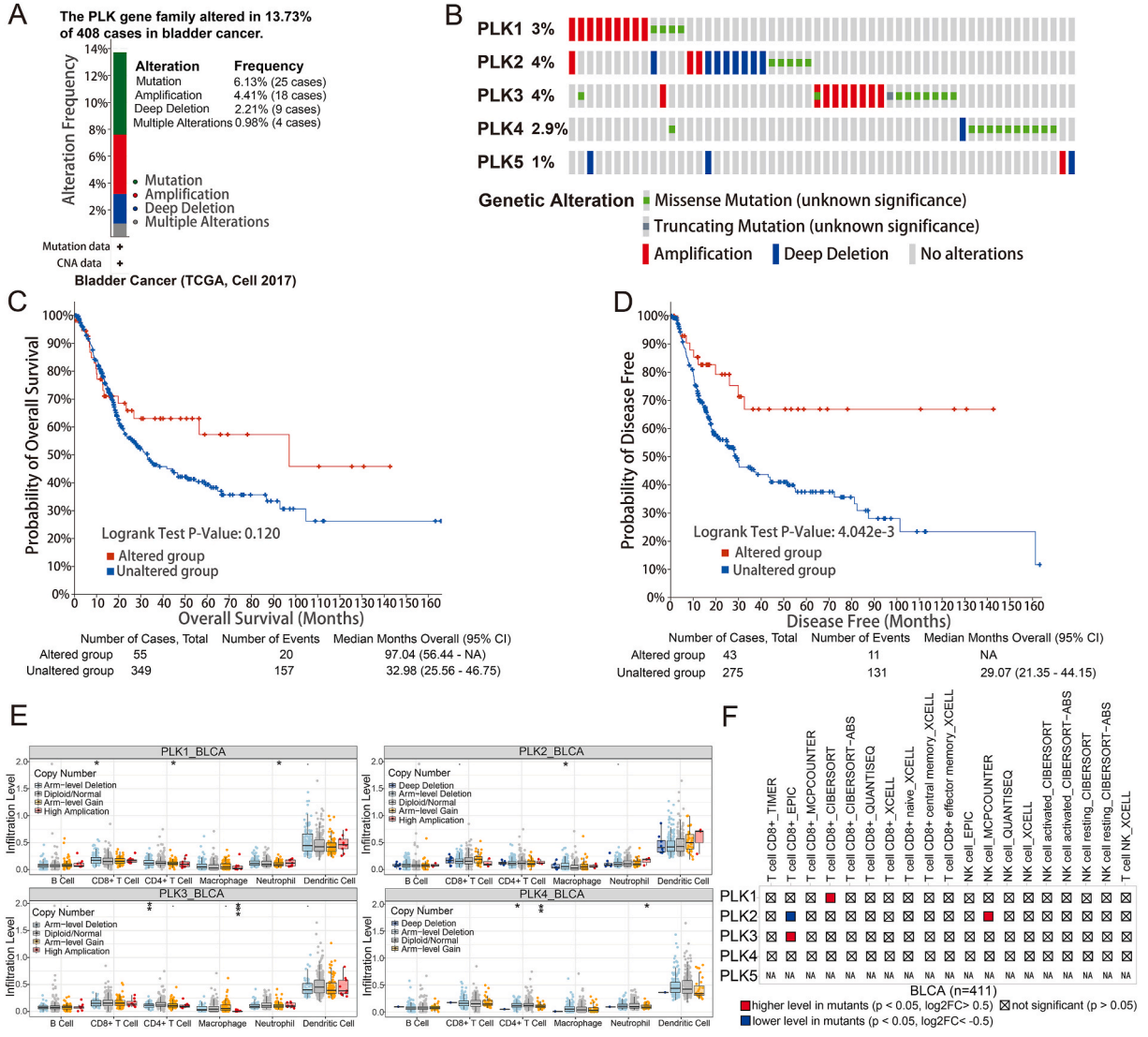

Fig. 4. Gene alternation of PLKs in bladder cancer was defined by cBioPortal database. **(A)** The overall changes of PLKs in bladder cancer included mutation, amplification, deep deletion, and multiple alternations. **(B)** The alternation frequency of each PLK genes was showed visually. **(C, D)** Kaplan-Meier curve was used to compare overall survival or disease-free survival with and without PLK alternations. The association between CNVs **(E)** or mutations **(F)** of PLK genes and immune infiltration in BLCA.

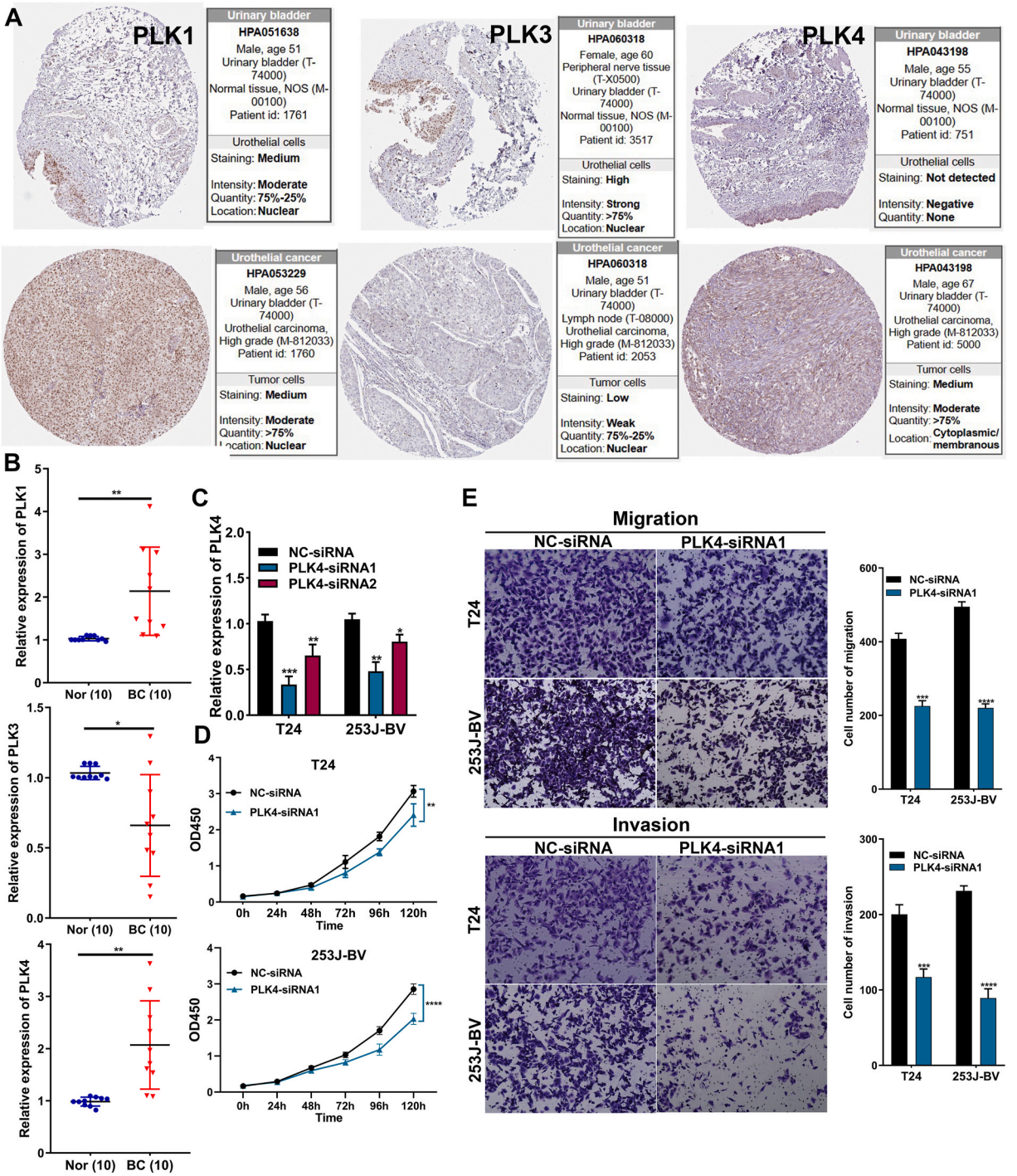

Fig. 5. Verification of PLKs expression in protein and mRNA level, as well as phenotype experiments after PLK4 knockdown. **(A)** Immunohistochemical staining for PLK1, PLK3 and PLK4 protein expression based on HPA database. **(B)** qRT-PCR result showed the mRNA expression level of PLK1, PLK3 and PLK4. **(C)** PLK4 small-interfering RNA (siRNA) transfection efficiency was assessed by qRT-PCR in T24 and 253 J-BV cells. **(D)** CCK-8 proliferation assay. **(E)** Migration and invasion were evaluated using a transwell assay without and with Matrigel, respectively. Data are showed as means ± SD; n = 3. SD, standard deviation; NC, negative control vector; CCK-8, cell counting kit-8; OD, optical density. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

Furthermore, the TCGA_BLCA alterations dataset suggests that ratio of deep deletion or amplification was lower versus shallow deletion or gain for PLKs from the TCGA investigation of entire genomes for bladder cancer, 2017. And, as the number of CNV copies increased PLK1/3/4 expression of genes dramatically increase (Supplementary Figs. S13A–E). The findings imply that ANXA upregulation in bladder tumors may be mainly caused by CNVs. Additionally, we noted that the proportion of the non-mutated area exceeded the proportion of the missense mutant. Among these, significant levels of matched expression of mRNA have been observed for missense PLK3 mutations whereas no significant relationship with the remaining genes has been identified (Supplementary Figs. S14A–E).

4.12. Relationship between PLK genomic variations and immune-cells infiltration in bladder cancer

To further explore the causes for the changes of PLK expression and immune microenvironment, we studied the relationship between the immunological microenvironment and CNVs in PLKs using data from the TIMER database (Fig. 4E). Single arm deletion of PLK1 increases $CD8^+$ T cells, while single arm gain of PLK1 decreases $CD4^+$ T cells and neutrophils. Moreover, single arm deletion of PLK2 and PLK3 enhances the macrophage content and $CD8^+$ T cells, respectively; whereas PLK3 amplification reduces macrophage content. For PLK4, it's single arm deletion increases $CD4^+$ T cells, but the single arm gain reduces $CD4^+$ T cells and neutrophils.

Additionally, mutations are another important factor for altered gene expression. Next, we analyzed the effect of PLKs mutations on immune cell content (Fig. 4F). PLK2 mutation decreases the content of $CD8^+$ T cells when compared to wild-type, however PLK3 mutations increase $CD8^+$ T cells. Furthermore, mutations in PLK1 and PLK2 increase the content of $CD8^+$ T cells and NK cells, respectively.

4.13. Validation of PLK genes expression in bladder cancer; the biological function of PLK4 in vitro

The protein expression of PLK1, PLK3, and PLK4 was found to be consistent with our prior analysis of the difference (Fig. 5A) after examining the HPA dataset. To further confirm PLK1/3/4 expression of genes by real-time PCR (RT-PCR), we chose ten pairs of BLCA tumors and cancer-adjacent healthy tissues (Fig. 5B). As our earlier data showed, PLK1/4 expression is elevated in cancerous tissue compared to expression in surrounding tissues, while PLK3 activity is decreased in cancerous tissue.

We also examined how PLK4 affected the biological activity of BLCA cells given the aforementioned findings. Using siRNAs against PLK4 or control siRNAs, we transiently transfected T24 and 253 J-BV cells for 48 h. By using qRT-PCR, the transfection effectiveness has been confirmed (Fig. 5C). The results of the CCK-8 studies showed that PLK4 interference dramatically decreased the T24 and 253 J-BV cell lines' ability to multiply when compared to NC (Fig. 5D). The transwell assay, carried out with or without Matrigel, as shown in Fig. 5E, consistently showed that PLK4 knockdown significantly decreased the T24 and 253 J-BV cell lines' ability to migrate and invade. Taking all considered, our results indicated that PLK4 is required for BLCA cell invasion, migration, and proliferation.

5. Discussion

The highly conserved class of serine/threonine protein kinases known as polo-like kinases, which are encoded by PLK family genes, is crucial for controlling mitotic checkpoints and cell division. It's have been considered as the research hotspots in cancer targeted therapy for their importance in cell division and mitotic checkpoint regulation. Within the realm of cancer studies, there has been a significant target on PLKs in recent years due to their involvement in a diverse array of biological processes, particularly cancer development. Numerous factors, including microRNAs, drug treatment, and gene mutations, etc [20–22], have been found to have a close association with PLKs, exerting a strong influence on tumor genesis and progression. Both single-targeting PLK inhibitors and dual-targeted therapy of PLK in conjunction with other cancer targets are regarded as promising approaches [23]. However, the majority of existing studies of the PLK family primarily focus on a single member or a single type of cancer. Investigating the association between the PLK family as a whole and pan-cancer patient prognosis or immunological properties remains largely unexplored. The present study employed a publicly accessible database to methodically evaluate the expression patterns of the five genes that comprise the PLK family. A comprehensive multi-omics analysis was conducted to investigate the expression of PLK genes in various kinds of cancer and possible links between these genes and the immune microenvironment in all cancer types, including BLCA.

Initially, we examined the expression of PLK family members in pan-cancer tissues and the variations in PLK family members' pattern of expression between adjacent non-cancerous tissues and cancerous tissues. Compared with other PLK family members, it is noteworthy that PLK2 has relatively high expression levels in pan-cancer tissues, and PLK5 exhibited significantly lower expression levels. Remarkably, various cancer types differ in the way that cancer and para-carcinoma tissues express different PLK family genes. Most members of the PLK family showed upregulated expression levels in various tumor tissues. This observation implies that the functional role of a PLKs ligand may vary according to the specific type of cancer or different members of the PLK family. For instance, while high expression of PLK1 has been reported in prostate and pancreatic cancers [24,25], Berus T et al. demonstrated that downregulation of PLK1 expression was linked to unfavorable prognosis in patients with uveal melanoma [26]. Additionally, the study by Abreu P et al. demonstrated that when PLK4 expression increased in HCC tissues, patients without microvascular infiltration had a favorable outcome [27]. The development of AML is highly correlated with PLK1 overexpression, and Berg T et al. have demonstrated that PLK2 is a known suppressor of tumor growth [28].

Subsequently, Using KM, multivariate, and univariate Cox regression analysis, we examined the clinicopathological traits of each member of the PLK family and assessed the potential predictive significance of PLK genes across different cancer types. We also examined the relationships between PLK family expression of genes in 33 malignancies and clinical or pathological characteristics or

prognosis (OS, DFS, DSS, and PFS). Furthermore, our findings demonstrated that compared with the diploid group, copy number gain/amplification and shallow/deep deletion might separately lead to the increased and decreased mRNA expression of PLK1/3/4 genes, respectively. The above results provided clues to understanding the potential role of PLKs and origin of variation in the PLKs in human cancer.

Next, it was observed that subtype C5 (immunologically quiet) exhibited relatively higher PLK5 expression than other subtypes. Whereas, the PLK1/2/3/4 genes show relatively lower expression in subtype C5. Research in related fields has made an identity for itself studying the interactions between malignancies and their environment. The presence of immune infiltration is closely linked to the advancement of tumor stages, and particular cellular constituents exert a substantial influence on the overall survival of patients [29]. The levels of PLK family and infiltration of immune cells, and even the immune score and stroma score in pan-cancer tissues, were found to be significantly associated in our study. Notably, the overexpression of PLK3 expression showed a clear positive link with the three parameters above in most forms of cancer, while PLK4/5 showed a negative correlation. These findings imply that PLK family genes might have a certain influence on the efficacy of immunotherapy. This may indicate that PLK3 overexpression may benefit pancreatic immune response, particularly cell-mediated immunity, conversely, PLK4/5 plays a negative regulatory role. Note that not all forms of cancer exhibit this pattern. For example, study by Jones W et al., PLK3 amplification was linked to USC metastasis and a poor outcome [30]. In our research, PLK1 exhibited a strong positive correlation with DNAss and RNAss and a significant negative association with immunological and stromal scores. Therefore, we speculated that upregulation of PLK1 may have an internal correlation with lower immune activity. Prior studies have shown that the expression of PLK1 is negatively linked with a variety of immune cell lineages, and inhibiting PLK1 could trigger the immune memory responses and modify a cancer immunological milieu by enhancing T cell invasion, promoting DC growth, and causing immunogenic cell death (ICD) [31]. The PLK1/PTEN axis in hepatocellular carcinoma is stimulated by the protein cell transformation sequence 2 (ECT2), leading to the polarization of M2 macrophages and a resulting suppression of NK and T cell activity [32]. However, PLK1 was found to be a negative regulator of transcriptional activity associated with NF-κB in recent times. Mammalian cells consistently express PLK1, which suppresses the expression of cyclin D1 and lessens the activation of IKK caused by tumor necrosis factor (TNF). As a result, there is less NF-κB activation and endogenous IkappaBalpha phosphorylation [33]. Zhou et al. revealed that PLK1/vimentin signaling facilitates metastasis and immune escape by triggering TGF-β/Smad 2/3 signaling to promote PD-L1 expression and may serve as predictive biomarkers for response to immunotherapy in LUAD [31]. It was found that PLK1 attenuates Pancreatic Cancer (PDAC) progression through inhibiting NFκB activity, and targeting PLK1 can stimulate anti-tumor immunity and potentiate the immunotherapy effectiveness by upregulation of PD-L1 in PDAC [34].

When paired with the matching ligands expressed on the surface of cancer cells, immunological checkpoints, specifically the programmed death receptor and its ligands, can prevent T lymphocytes from killing cancer cells. This has significant consequences for the immune escape mechanism of tumors [35]. We investigated the interaction between the 47 common immune checkpoint genes (including PD-1, PD-L1, CTLA-4, etc.) and PLK family genes. The findings showed that immune checkpoint expression across nearly all cancer types was positively or negatively related to the expression of the majority of PLK gene family members. Practically each immunological checkpoint and PLK3 expression have a strong positive correlation. However, the PLK5 expression was inversely correlated with the expression of the immune checkpoint. In addition, a statistically significant link was found across a variety of pan tumor between the PLK family members expression and the MSI or TMB. For instance, across several tumor species, we discovered that high PLK1 expression was positively connected with both MSI and TMB, which is in line with the findings of the Qian Y et al. study [36]. This finding implies that, in certain types of cancer, the expression of PLK3 is adversely associated with TMB; in contrast, PLK4/5 has a strong positive correlation. Overall, our research indicates that some PLK family members may be highly useful in predicting the effectiveness of tumor immunotherapy.

To fill the PLK study in the field of bladder cancer, we specifically analyzed the PLK family in BLCA. First, we conducted the KEGG pathway enrichment analysis of each PLK member in bladder tumor and discovered that several pathways linked to cancer have been improve. “Moreover, we have also demonstrated that PLK family members were significantly associated with immune subtypes in BLCA. Expression of PLK2/3 had a clearly negative correlation with stem cell score (i.e., RNAss or DNAss), nevertheless, PLK1/4/5 showed significant positive correlations. Most members of the PLK family (including PLK2, PLK3, PLK4 and PLK5) represented significant negative or positive correlations with Immune, Stromal, or ESTIMATE scores. Specifically, PLK3 expression displayed a positive correlation with immune score, stromal score, and ESTIMATE score, whereas PLK5 expression exhibited a negative correlation with these three scores. Besides, using TIMER platform, we further determined significant correlations of PLK genes with immune cell infiltration of the tumor microenvironment, such as $CD8^+$ T, $CD4^+$ T, Neutrophil, Dendritic cells. Finally, we examined the relationship between the infiltration of immune cells in BLCA and the genetic variations of PLKs, and found that several of the copy number variation had relatively lower infiltration of immune cells than diploid/normal copy number, except for PLK2 arm-level deletion.” Our results also show that the PLK1/2/3 mutations versus the quantity of immunological cells in the tissues of BLCA cancers may not be impacted by any alterations.

In our analysis, PLK family genes, particularly PLK3, exhibited evident patterns across various cancers in terms of both transcriptional and genetic modifications, as well as its significant correlation with immune cells infiltration. The alteration in PLK3’s transcriptional activity could potentially arise from gene amplification, shallow deletion, or acquisition. The correlation analyses of PLK3 expression with immunological characterization (including immune typing, immune score, stromal score, ESTIMATE score and immune checkpoints, etc) showed that tumor patients with high PLK3 expression might contribute to patients’ favorable therapeutic outcomes and prognosis, which indicates that PLK3 may have important clinical implications as a tumor suppressor [37]. The involvement of PLK5 in monitoring the extent of immune infiltration across various types of cancer and providing guidance for immunotherapeutic interventions in pan-cancer settings is of considerable importance. The kinase PLK5 plays a crucial role in

monitoring the extent of immune infiltration in pan-cancer cases and providing guidance for pan-cancer immunotherapeutic interventions. Additional investigation into bladder cancer has further validated the importance of PLK5, thereby suggesting a strong association between PLK5 and immune response.

This research presents a comprehensive integrative multi-omics analysis for PLK family based on multiple datasets. Specifically, we conducted a systematic integrated analysis of PLK family in transcriptome expression and its alteration in pan-cancer. Additionally, we investigate the association between PLK family expression and immune checkpoint, sensitivity to chemotherapy agents, tumor microenvironment, and immunotherapy efficacy. These findings provide valuable guidance for clinical practice. Furthermore, a comprehensive analysis of PLKs was also conducted to elucidate their role in bladder cancer. It was observed that PLK genes may function as tumor regulators and hold potential as diagnostic and prognostic biomarkers in BLCA patients. Additionally, our investigation revealed that the PLK family is enriched in various pathways associated with cancer, providing valuable insights. This was confirmed through the use of qRT-PCR, HPA validation, and cell phenotype experiments. Notably, some members of PLK family might act as predictors of the efficacy of immunotherapy. The specific mechanisms and significance of these PLK family genes should also be commonly discussed in future.

Furthermore, FDR adjustments for various comparisons were employed to streamline the evaluation and interpretation of significant results. The findings indicate that a number of the PLKs' notable discoveries may be false positives, therefore caution should be exercised when interpreting them. Nonetheless, the bulk of significant results are still quite robust even after FDR adjustment (FDR <0.05). Overall, the study's findings will remain unaffected by these modifications. The research is limited by the absence of functional experiments to elucidate the relevant molecular mechanisms of PLK genes, despite a series of integrative analyses of multiomics data from open databases (e.g., TCGA, GEO, and cBioPortal), the PCR histological validation, and preliminary in vitro experiments of PLK4. Additionally, we conducted a somewhat complicated analysis of the PLK family's numerous omics data spanning pan-cancer.

6. Conclusion

In summary, our study demonstrated that PLK family genes, from a systems-wide perspective of multi-omics and multi-datasets, was highly related to survival and prognosis. Moreover, the correlation between PLKs and immune infiltration, immunotherapy response displayed all that the expression of PLKs could be used to guide treatment. The critical involvement of PLKs in bladder cancer was also demonstrated by more research, validation, and in vitro tests of PLKs. However, more investigation continues to be needed to confirm in the future.

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Ethics approval and consent to participate

This research has been conducted in accordance with the Declaration of Helsinki and has been approved by the ethics committee of the 2nd Affiliated Hospital of Tianjin Medical University (Ethics code, KY2021K003) with proper written documentation of informed consent.

Data availability statement

The data were obtained from the TCGA and GEO online repositories. Data supporting the findings presented in the study are included in the paper and its supplementary information files. Further inquiries can be directed to the corresponding authors.

CRediT authorship contribution statement

Chong Shen: Conceptualization, Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – original draft, Methodology. **Tong Wang:** Data curation, Formal analysis, Software, Writing – original draft. **Kai Li:** Data curation, Investigation, Methodology, Software, Writing – review & editing. **Chong Fu:** Data curation, Investigation, Methodology, Resources, Software, Validation. **Shaobo Yang:** Investigation, Methodology, Resources, Validation. **Zhe Zhang:** Formal analysis, Investigation, Visualization. **Zhouliang Wu:** Data curation, Formal analysis, Funding acquisition, Investigation, Validation. **Zhi Li:** Conceptualization, Investigation, Methodology. **Zhuolun Li:** Formal analysis, Investigation, Resources, Software. **Yuda Lin:** Conceptualization, Data curation, Formal analysis, Software. **Yu Zhang:** Data curation, Formal analysis, Funding acquisition, Investigation. **Jian Guo:** Data curation, Investigation, Methodology, Software. **Zhenqian Fan:** Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **Hailong Hu:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this chapter can be found online at https://doi.org/10.1016/j.heliyon.2024.e28048.

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