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Targeting P21-activated kinase suppresses proliferation and enhances chemosensitivity in T-cell lymphoblastic lymphoma

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Abstract

T-cell lymphoblastic lymphoma (T-LBL) is a highly aggressive non-Hodgkin lymphoma with a poor prognosis. P21-activated kinase (PAK) is a component of the gene expression-based classifier that can predict the prognosis of T-LBL. However, the role of PAK in T-LBL progression and survival remains poorly understood. Herein, we found that the expression of PAK1 was significantly higher in T-LBL cell lines (Jurkat, SUP-T1, and CCRF-CEM) compared to the human T-lymphoid cell line. Moreover, PAK2 mRNA level of 32 relapsed T-LBL patients was significantly higher than that of 37 cases without relapse (P = .012). T-LBL patients with high PAK1 and PAK2 expression had significantly shorter median RFS than those with low PAK1 and PAK2 expression (PAK1, P = .028; PAK2, P = .027; PAK1/2, P = .032). PAK inhibitors, PF3758309 (PF) and FRAX597, could suppress the proliferation of T-LBL cells by blocking the G1/S cell cycle phase transition. Besides, PF could enhance the chemosensitivity to doxorubicin in vitro and in vivo. Mechanistically, through western blotting and RNA sequencing, we identified that PF could inhibit the phosphorylation of PAK1/2 and downregulate the expression of cyclin D1, NF-κB and cell adhesion signaling pathways in T-LBL cell lines. These findings suggest that PAK might be associated with T-LBL recurrence and further found that PAK inhibitors could suppress proliferation and enhance chemosensitivity of T-LBL cells treated with doxorubicin. Collectively, our present study underscores the potential therapeutic effect of inhibiting PAK in T-LBL therapy.

Key Words: P21-activated kinase; PAK inhibitor; Proliferation; Relapse; T-cell lymphoblastic lymphoma

1. INTRODUCTION

T-cell lymphoblastic lymphoma (T-LBL) is a rare subtype of non-Hodgkin lymphoma derived from precursor lymphoblastic cells.[1] T-LBL and T-cell acute lymphoblastic leukemia (T-ALL) are grouped together in the current World Health Organization (WHO) classification. Although ALL-like intensive chemotherapy has improved the response rate of T-LBL, about 10% to 20% of T-LBL patients remain resistant to chemotherapy.[2,3] Unlike pediatric patients, adult T-LBL patients have a lower tolerance to intensive chemotherapy regimens, which may cause a reduced-dose intensity of induction therapy and consequently affect the response rates and survival outcome.[4] In addition, high-risk adult T-LBL patients also experience high recurrence rates (40%–60%) and dismal prognoses after allogeneic or autologous hematopoietic stem cell transplantation (HSCT).[5,6] Nevertheless, there is currently no targeted therapy approved for T-LBL by the US Food and Drug Administration (FDA).[7,8] Therefore, it is necessary to explore novel therapeutic targets to improve the effectiveness of conventional chemotherapeutic agents in T-LBL.

Kinases are common drivers of malignancies and are considered potential targets for anti-tumor therapy. Several readily manufactured kinase inhibitors are effective and have been approved for the treatment of various malignancies. For instance, the combination of kinase inhibitors and standard chemotherapy has significantly improved the outcomes of Philadelphia chromosome-positive ALL patients.[9] We previously constructed several prognostic prediction models for adult T-LBL using miRNA, CpG methylation and gene expression levels.[10–12] Notably, p21-activated kinase (PAK) was identified in the gene expression model of adult T-LBL, implying that PAKs might have important biological roles in T-LBL. PAKs are members of serine/threonine kinases

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composed of six subtypes, which are divided into two groups based on the sequence and structural homology: group I PAKs (including PAK1, PAK2, and PAK3) and group II PAKs (including PAK4, PAK5, and PAK6).[13] Biologically, PAKs participate in various cellular processes, including cell growth, cell survival, cytoskeleton remodeling, cell migration, and cell morphology.[14] PAKs are frequently upregulated in various malignancies and influence tumor cell proliferation, signal transduction, tumor invasion, drug resistance, and immune regulation,[15,16] making PAKs the confluence of several oncogenic signaling pathways. However, the biological function and mechanism of PAKs and the therapeutic activity of PAK inhibitors in T-LBL remain largely unknown.

The current study aimed to evaluate the expression, biological function, and mechanism of PAKs in T-LBL. We also assessed the potential anti-tumor activity of PAK inhibitors (PAKi) in T-LBL cells in vitro and in vivo, either as a single agent or in combination with a chemotherapeutic drug.

2. MATERIALS AND METHODS

2.1 Cell culture

The cell lines used in this study included the human T-LBL cells Jurkat (ATCC, Manassas, Virginia), SUP-T1 (ATCC), CCRF-CEM (BeiNa Biotechnology, Beijing, China), and the human immortalized T-lymphocyte H9 (BeiNa Biotechnology). All cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco, ThermoFisher, Waltham, Massachusetts) supplemented with 10% fetal bovine serum (FBS; Biological Industries, 304-001-1ACS) and 1% penicillin/streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. All cell cultures were routinely tested for mycoplasma contamination.

2.2 Patient and tissue samples sequence

Formalin-fixed and paraffin-embedded (FFPE) tissue samples from 69 T-LBL patients were obtained from Sun Yat-sen University Cancer Center (SYSUCC). Details of the inclusion and exclusion criteria were described in our previous studies.[10–12] The expression of PAKs was detected by Affymetrix human gene 2.0 ST microarray (Thermo Fisher Scientific, Waltham, Massachusetts) as previously described.[11] The study protocol (B2020-046) was approved by the institutional review board of SYSUCC.

2.3 Cytotoxicity assay

PF3758309 (PF, Selleckchem, Houston, Texas, Cat. no. S7094) is a potent ATP-competitive inhibitor that targets PAK1, PAK2, PAK3, PAK4, PAK5, and PAK6. FRAX597 (Selleckchem, Cat. no. S7271) is a selective ATP-competitive group I PAK inhibitor that binds to PAK1, PAK2, and PAK3. Both PAKi were dissolved in dimethyl sulfoxide (DMSO). The DNA topoisomerase II inhibitor, doxorubicin (DOX, Selleckchem, Cat. no. S1208), was used to evaluate the synergetic effects of PAKi and chemotherapy. Doxorubicin was dissolved in water. Cells were exposed to various concentrations of the reagents for 48 hours, and the cell viability was measured using the CCK-8 assay kit (DOJINDO, Cat. no. CK04-05). The 50% inhibitory concentrations (IC_{50}) were calculated using Graph Pad Prism 7 (Graph Pad Software Inc., San Diego, California).

2.4 Flow cytometry-based apoptosis and cell cycle assay

Cells were treated with various concentrations of PAKi or DOX for 24 hours. Cell apoptosis and cell cycle were detected by flow cytometry according to standard protocols provided by the manufacturer (Annexin V-APC/PI cell apoptosis detection kit, KeyGEN BioTECH, Cat. no. KGA1030; Cell cycle kit KeyGEN BioTECH, Cat. no. KGA512).

2.5 Quantitative real-time polymerase chain reaction

Total RNA was isolated using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany). RNA was quantified using absorbance at a wavelength of 260 nm, and 1 μg of total RNA was used for cDNA synthesis with PrimeScript RT Master Mix kit (Takara, Kyoto, Japan, Cat. no. RR036A). Real-time polymerase chain reactions (PCR) were carried out using the Takara SYBR Premix Ex Taq kit (Takara, Cat. no. RR420) in the LightCycler 480 Instrument II (Roche, Basel, Switzerland). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Ct, cycle threshold) relative to GAPDH. The corresponding primers are presented in Supplemental Table S1, http://links.lww.com/BS/A67.

2.6 Western blotting

Western blotting was performed as previously described.[15–17] Primary antibodies specific to PAK1, PAK2, PAK3, PAK1/2/3, p-PAK1(Ser144), p-PAK1(Ser188/204), p-PAK1(Thr423), p-PAK2(Ser141), p-PAK2(Thr402), p-PAK(Ser20), NF-κ, cyclinD1 (1:1000, Cell Signaling Technology, Danvers, Massachusetts) and GAPDH (1:1000, Abcam, Cambridge, UK) were used. The blots were then incubated with goat anti-rabbit secondary antibodies (Promega, Madison, Wisconsin) and visualized using enhanced chemiluminescence.

2.7 Immunohistochemical staining

Briefly, the methods for immunohistochemical staining were performed as described in our previous study.[15–17] All prepared slides underwent deparaffinization and rehydration with standard xylene-to-ethanol washes. The slides were then blocked in phosphate-buffered saline (PBS) with 0.025% Tween 20 with 10% FBS. After blocking, the slides were incubated at 4°C overnight with primary antibodies. Negative controls were prepared by substituting PBS for the primary antibodies. The following primary antibodies were used: anti-PAK1 + PAK2 + PAK3 (phospho S144 + S141 + S154) (1:100; ab40795; Abcam), anti-PAK1 + PAK2 + PAK3 (phospho T423 + T402 + T421) (1:100; ab62155; Abcam,), anti-cyclin D1 (1:1000; 26399-1-1p; Proteintech, Wuhan, China), anti–NF-Kb (1:800; 8242; Cell Signaling Technology), and anti–Ki-67 (1:2000; 05298512001; Roche). Next, the slides were incubated with HRP-conjugated anti-rabbit IgG secondary antibodies (Cell Signaling Technology, Cat. no.8114) for 30 minutes.

2.8 RNA sequencing analysis

Jurkat and SUP-T1 cells were treated with PF ($0.5 \times IC_{50}$) or DMSO for 24 hours. Then, total RNA was extracted from cells using TRIzol (Invitrogen). Library construction and sequencing were performed by Annoroad Gene Technology (Beijing, China). The libraries were sequenced on an Illumina HiSeq 2500 platform, and 100-bp paired-end reads were generated. The reference genomes and the annotation file were downloaded from the ENSEMBL database (http://www.ensembl.org/index.html). Bowtie2 v2.2.3 was used for building the genome index, and Clean Data was then aligned to the reference genome using HISAT2 v2.1.0. DEGseq was used for differential gene expression analysis. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed based on RNA-seq data and using DAVID v6.8.[18] The top significantly enriched GO, and KEGG terms were selected for plotting.

2.9 Xenograft studies

In tumor formation assay, 1×10^7 SUP-T1 cells were suspended in 100 μL of PBS/Matrigel (1:1) mixture and injected subcutaneously into the right flanks of 4-week-old BALB/C-nu/nu athymic nude mice (Guangdong Medical Laboratory Animal

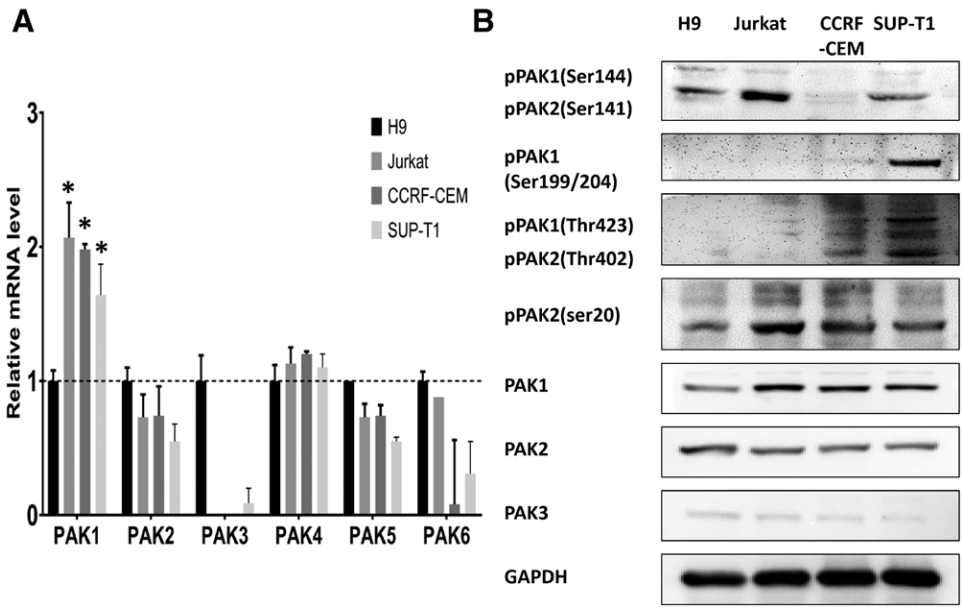

Figure 1. PAK expression patterns in human T-LBL cells. (A) The PAK mRNA levels in the H9 cell and T-LBL cells were detected using quantitative real-time PCR. (B) Western blotting detection of PAK protein levels in H9 cell and T-LBL cells. T-LBL cells: Jurkat, SUP-T1, CCRF-CEM. The human immortalized T-lymphocyte cell H9 was used as the control. *P < .05. PAK = P21-activated kinase, PCR = polymerase chain reaction, T-LBL = T-cell lymphoblastic lymphoma.

Center, Guangzhou, China). Animals were randomly assigned into four groups: control group, treated with PBS once daily; PF group, treated with 10 mg/kg PF once daily; DOX group, treated with 2 mg/kg doxorubicin once daily; combination group, treated with PF and doxorubicin once daily. All interventions were administered intraperitoneally. PF and DOX treatment was initiated when tumors reached ~100 mm^3. Throughout the experiment, the overall health of the animals was closely monitored. After day 14 of treatment, the mice were euthanized, and tumors were dissected. Then the tumor weight was recorded. The in vivo study was approved by the Animal Care and Use Committee of Sun Yat-sen University Cancer Center (Guangzhou, Guangdong, China). All experiments were carried out strictly following the established institutional guidelines and the USA National Institutes of Health (NIH) guidelines for using experimental animals.

2.10 Statistical analyses

Data are presented as the mean ± SD from three independent experiments. Two-tailed Student t tests and one-way analysis of variance (ANOVA), followed by Dunnett tests for multiple comparisons, were used to evaluate the data. The combination index (CI) was calculated using the equation CI = AB/(A × B).[19] CI < 1 indicates a synergistic effect. Relapse-free survival (RFS) time was calculated using the Kaplan-Meier method, and differences were assessed using the log-rank test. Pearson chi-square and Fisher exact tests were used to determine the association between clinical characteristics and PAK1/2 expression. All statistical analyses were performed with SPSS 24.0 or GraphPad Prism 7.0 software. A difference was considered to be statistically significant when P value <.05.

3. RESULTS

3.1 Expression patterns of PAK family members in T-LBL cells

First, we evaluated the expression level of the PAK isoforms in T-LBL cell lines. PAK1 mRNA and protein levels in T-LBL cells (Jurkat, SUP-T1 and CCRF-CEM) were significantly higher than that of human immortalized T-lymphocyte cells (H9; **Fig. 1A, B**). We also evaluated the phosphorylation of PAK in the T-LBL cells, and different phosphorylated sites were identified, including p-PAK2 (Ser141) and p-PAK2 (Ser20) in the Jurkat cell line, p-PAK1 (Thr423), p-PAK2 (Thr402) and p-PAK2 (Ser20) in CCRF-CEM cell line, p-PAK1 (Ser199/204), p-PAK1 (Thr423), p-PAK2 (Ser141), p-PAK2 (Thr402) and p-PAK2 (Ser20) in SUP-T1 cell line (**Fig. 1B**).

3.2 High PAK1/2 mRNA level was associated with recurrence of T-LBL

To investigate the expression and prognostic value of PAK isoforms in T-LBL patients, the mRNA expression profile of 69 T-LBL tissue samples was established and analyzed. Among the 69 T-LBL patients, 44 (63.8%) were male, and the median age at diagnosis was 30 years (16–44 years). Most patients (72.5%) received T-ALL-like chemotherapy. As shown in Figure 2A, the PAK2 mRNA level of 32 relapsed T-LBL patients was significantly higher than that of 37 patients with no relapse (P = .012); but there was no difference among PAK1, 3, 4, 5/7 mRNA expression. The upper quartile (P75) was defined as the high expression cut point of PAK1 and PAK2. Overall, patients with high PAK1 or PAK2 mRNA expression levels had worse median RFS (PAK1, median RFS 31.5 months vs not reached [NR], hazard ratio [HR] = 3.001 P = .028; PAK2, median RFS 25.7 months vs NR, HR = 3.981, P = .027) (**Fig. 2B, C**). Using the median as the boundary value, the high expression of both PAK1 and PAK2 was defined as PAK1/2 high expression. Notably, T-LBL patients with high PAK1/2 mRNA expression developed earlier recurrence than patients with low PAK1/2 mRNA expression (median RFS 26.0 months vs NR, HR = 2.721, P = .032) (**Fig. 2D**). Additionally, patients with high PAK1/2 mRNA expression were prone to worse clinicopathological characteristics, including low hemoglobin level, high Ki-67 expression, pleural/pericardial effusion, bone marrow involvement and low CR rates (P < .05;).

Table 1

Relationship between PAK1/2 mRNA expression and clinical characteristics in T-LBL patients.

Clinical characteristics	PAK1/2 mRNA		*P*
	Low (n = 21)	High (n = 48)	
Age (y), No. (%)			.733
≥30	11 (52.4)	23 (47.9)	
<30	10 (47.6)	25 (52.1)	
Gender, No. (%)			.740
Male	14 (66.7)	30 (62.5)	
Female	7 (33.3)	18 (37.5)	
ECOG-PS, No. (%)			.599
≥2	7 (33.3)	13 (27.1)	
<2	14 (66.7)	35 (72.9)	
Fever, No. (%)			.740
Yes	3 (14.3)	10 (20.8)	
No	18 (85.7)	38 (79.2)	
HGB (G/L), No. (%)			.042*
≥130	11 (52.4)	13 (27.1)	
<130	10 (47.6)	35 (72.9)	
LDH (U/L), No. (%)			.219
≥245	13 (61.9)	22 (45.8)	
<245	8 (38.1)	26 (54.2)	
Ki-67, No. (%)			.017*
≥70%	8 (38.1)	33 (68.8)	
<70%	13 (61.9)	15 (31.3)	
Effusion, pleural/pericardia, No. (%)			.050*
Yes	4 (19.0)	21 (43.8)	
No	17 (81.0)	27 (56.3)	
Mediastinal involvement			.698
Yes	12 (57.1)	25 (52.1)	
No	9 (42.9)	23 (47.9)	
Bone marrow involvement, No. (%)			.010*
Yes	8 (38.1)	34 (70.8)	
No	13 (61.9)	14 (29.2)	
Tumor burden (cm), No. (%)			.069
≥10	4 (19.0)	20 (41.7)	
<10	17 (81.0)	28 (58.3)	
Ann Arbor stage, No. (%)			.712
I–II	2 (9.5)	7 (14.6)	
III–IV	19 (90.5)	41 (85.4)	
Complete response, No. (%)			.020*
Yes	16 (76.2)	22 (45.8)	
No	5 (23.8)	26 (54.2)	

*$P < .05$

ECOG-PS = Eastern Cooperative Oncology Group performance status, HGB = hemoglobin, LDH = lactate dehydrogenase, PAK = P21-activated kinase.

3.3 PAKi inhibited cell proliferation by regulating the cell cycle in T-LBL

Given the expression pattern of the PAK family in T-LBL, two PAK inhibitors (PaKi), PF3758309 (PF) and FRAX597, were selected for PAK pharmacological inhibition. CCK-8 assays revealed that both PAKi could significantly inhibit cell viability in a dose-dependent manner in all three T-LBL cell lines after a 48-hour treatment window (**Fig. 3A**). All three T-LBL cell lines showed high sensitivity to PF, and IC_{50} values of the two PAK inhibitors are shown in Figure 3B. Next, we evaluated the pharmacological effects of PF in a SUP-T1 xenograft mouse model with daily intraperitoneal administration of 10 mg/kg PF. Notably, PF could significantly attenuate tumor growth, with an average of 30.5% reduction in tumor weight after a 2-week treatment regimen (**Fig. 4B**).

Flow cytometry assays were performed to determine whether PAKi could influence cell cycle regulation or induce cell apoptosis. After treatment using PF and FRAX597 at IC_{50} concentration for 24 hours, all three T-LBL cells showed significant G_0/G_1 cell cycle arrest, while the S phase transition was promoted (**Fig. 3D**). Treatment with PF for 24 hours also induced significant dose-dependent apoptosis in Jurkat and CCRF-CEM cells but not in SUP-T1 cells (**Fig. 3C**). Taken together, these results indicate that PAK inhibition could suppress the proliferation of T-LBL cells via regulating the G1/S phase transition.

To explore the function of PAKs in T-LBL, we established PAK1-knockdown SUP-T1 cells and PAK2-knockdown SUP-T1 cells, and qRT-PCR and western blot was performed to confirm the efficiency (Supplemental Figure S1A–B, http://links.lww.com/BS/A67). CCK-8 assays showed that the proliferation of SUP-T1 cells were significantly decreased after PAK1 and PAK2 silencing (Supplemental Figure S1C, http://links.lww.com/BS/A67). Furthermore, flow cytometry assays demonstrated that PAK1- and PAK2-knockdown both increased the percentage of cells in G0/G1 phase and reduced the percentage of cells in S phase in SUP-T1 cells (Supplemental Figure S1D–E, http://links.lww.com/BS/A67). These data indicated that both PAK1 and PAK2 enhanced proliferation via regulating G1/S phase transition.

3.4 PF enhanced the chemosensitivity of doxorubicin in T-LBL in vitro and in vivo

Intensive chemotherapy using drugs such as doxorubicin (DOX) is fundamental in the treatment of T-LBL. However, chemotherapy resistance and recurrent disease are challenging obstacles that need to be overcome.[9] To evaluate whether the addition of PAKi could enhance the anti-tumor activity of doxorubicin, three T-LBL cell lines were treated with DOX and PF simultaneously at various concentrations for 48 hours according to Punnett square.[20] Compared with treatment with DOX or PF alone, the combination of DOX and PF was more effective. The combination index (CI) values were 0.505, 0.560, and 0.564 in Jurkat, CCRF-CEM, and SUP-T1 cells, respectively. Collectively, PF and DOX had a synergistic anti-proliferative effect in all three T-LBL cells in vitro (**Fig. 4A**). In the SUP-T1 xenograft model, treatment with PF also significantly increased the sensitivity of T-LBL to DOX treatment, as indicated by the decreased tumor volume and weight (**Fig. 4B**). Together, the above data demonstrate the synergistic anti-tumor effect of PAKi and DOX in vitro and in vivo, and this combination regimen may represent a novel strategy in the treatment of T-LBL patients.

3.5 Identification of the target genes and pathways of PF in T-LBL

Both PAK kinase-dependent and independent activities are important to its function. Hence, total PAK protein and phosphorylated PAK protein were detected in T-LBL cells treated with PF at two concentrations (IC_{50} and $2 \times IC_{50}$) for 24 hours. Western blotting revealed significant downregulation of phosphorylated PAK proteins, especially p-PAK2 (Ser141) and p-PAK2 (Ser-20) (**Fig. 5A**). This shows that PAKi could reduce T-LBL cell viability by downregulating PAK phosphorylation. To evaluate the effect of PAKi on other targets, the cell cycle-related protein cyclin D1 and proliferation-related protein NF-κB were detected in Jurkat cells by western blotting. After being treated with PF for 24 hours, the protein level of cyclin D1 and NF-κB were downregulated, as well as that of phosphorylated PAK1/2 (**Fig. 5B**). Immunohistochemical staining of xenograft tumor samples showed that the phosphorylation level of PAK, NF-KB, and cyclinD1 also decreased following treatment with PF (**Fig. 5C**). To further explore the molecular mechanism by which PAK inhibitors exert their biological effects, genome-wide RNA sequencing was conducted to compare gene expression profiles between T-LBL cells treated with and without PF. Interestingly, there were 1344 and 784 differentially downregulated genes in Jurkat and SUP-T1 cell lines after PF treatment, respectively (**Fig. 5D**). A total of 159 common downregulated genes in both Jurkat and SUP-T1 cells were further analyzed. GO and KEGG enrichment analysis of these 159 downregulated genes revealed

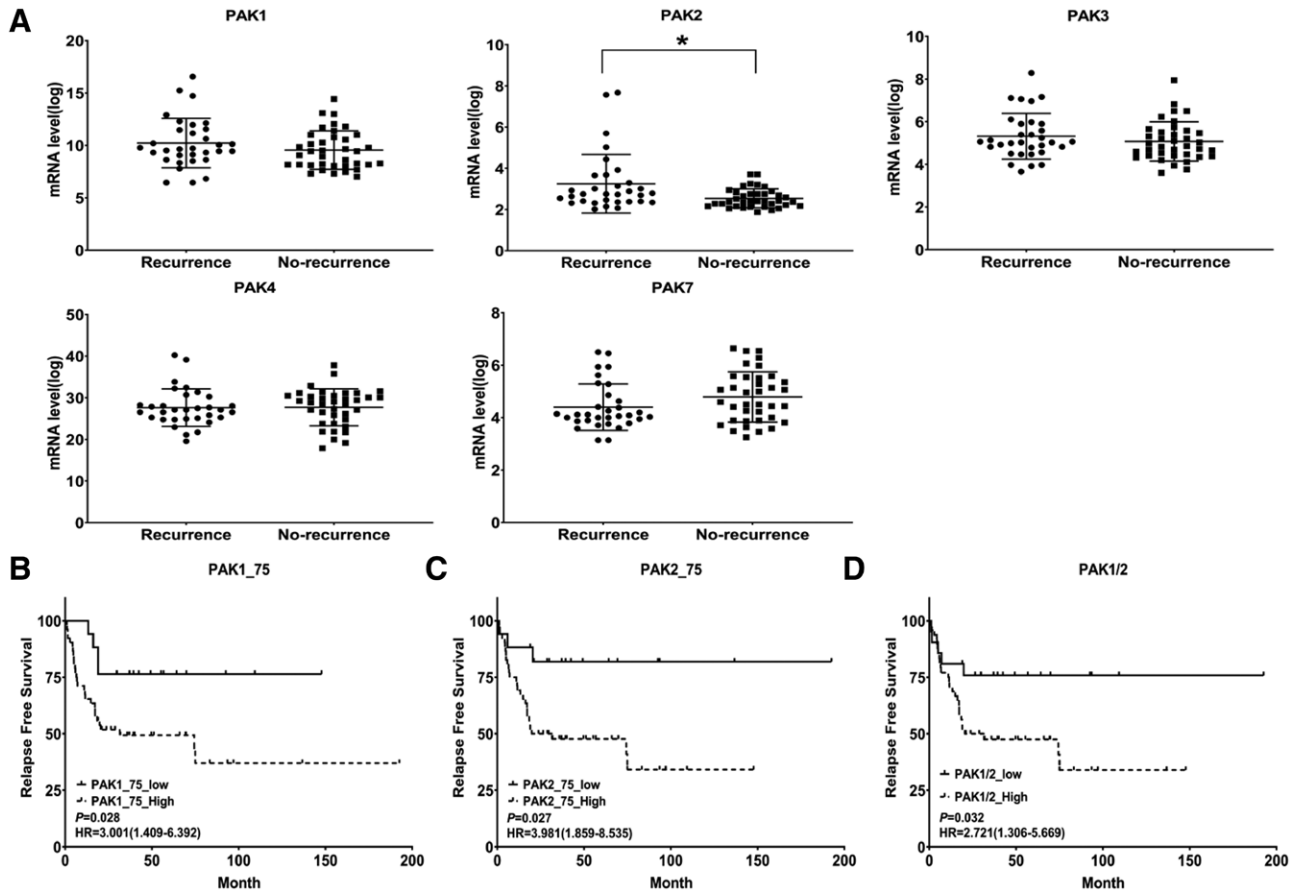

Figure 2. High PAK1/2 mRNA level was correlated with recurrence of T-LBL. (A) Relationship between PAKs mRNA expression and recurrence in T-LBL patients; (B) RFS survival curves in T-LBL patients with high and low PAK1 mRNA levels (median RFS 31.5 mo vs NR, HR = 3.001, *P* = .028); (C) RFS survival curves of T-LBL patients with high and low PAK2 mRNA levels (median RFS 25.7 mo vs NR, HR = 3.981, *P* = .027); (D) RFS survival curves of T-LBL patients with high and low PAK1/2 mRNA levels (median RFS 26.0 mo vs NR, HR = 2.721, *P* = .032). **P* < .05. NR = not reached, PAK = P21-activated kinase, RFS = relapse-free survival, T-LBL = T-cell lymphoblastic lymphoma.

that PF could influence the expression of serial genes related to cell adhesion and other essential pathways (**Fig. 5E**).

4. DISCUSSION

Identifying potential therapeutic targets and novel targeted agents is urgently needed for T-cell lymphoblastic lymphoma patients. To that end, our study identified overexpressed PAK family members in T-LBL cells and tissues and demonstrated the potential prognostic value of PAK1/2 expression level in T-LBL patients. Moreover, we found that PAKi could suppress T-LBL cell proliferation through G1/S transition inhibition and enhance the sensitivity of the chemotherapeutic drug DOX in T-LBL. Further research into the mechanism of PAKi-induced tumor suppression revealed that PF inhibited phosphorylate PAK1/2 and other target genes and pathways such as cyclin D1 and NF-κb. Taken together, our findings emphasize the biological role of PAK and the potential therapeutic value of PAKi in T-LBL treatment.

Alterations (including amplifications and mutations) of PAKs are frequently found in different cancers.[13] Studies have found that PAK1, PAK2, and PAK4 are generally expressed in most cancer types, while the other four isoforms exhibited a tissue-specific expression.[21] Among the hematological malignancies, 24% of adult T-cell leukemia/lymphoma (ATLL) patients exhibited PAK2 gene amplification.[22] Meanwhile, PAK1 overexpression is found in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS).[23] In the present study, PAK1 was highly expressed in T-LBL cell lines, and the phosphorylation sites of PAK1 and PAK2 varied among the three T-LBL cell lines. PAK overexpression is commonly associated with cancer progression, acting as a prognostic biomarker in various cancers, including ATLL, AML, etc.[22–24] However, in contrast to the oncogenic function of other PAKs, PAK6 has both oncogenic and tumor suppressive functions depending on the cancer type.[25,26] Our study found that the expression of PAK2 was increased in patients with recurrent T-LBL, and the overexpression of PAK1/2 mRNA may be associated with earlier relapses in T-LBL patients. T-LBL patients with high PAK1/2 mRNA levels had more adverse clinicopathologic features, including bone marrow involvement and inferior response to induction chemotherapy. The deregulated expression pattern and correlation with prognosis highlight the importance of understanding the biological role of PAKs in T-LBL.

PAKs and their activators and substrates have been shown to be involved in regulating cell proliferation, cell growth, angiogenesis, metastasis, and drug resistance, indicating a critical role for PAKs in cancer progression. PAK and other cell cycle-related proteins, such as cyclins, can directly or indirectly regulate cell cycle processes. Through inhibiting the MAPK/PI3K pathway, PAK1 could upregulate cyclin D1 expression and induce cell cycle transition from G1 to S phase in hepatocellular carcinoma.[27] PAK4 also participates in cell cycle regulation via p21 in breast cancer.[28] Our study also found that cyclin D1 is downregulated by PAKi, inducing cell cycle arrest in T-LBL cells. Besides, PAKs can also act as the kinase of NF-κB to activate the NF-κB signaling pathway, leading to cancer development.[29,30] Notably, phosphorylation of PAK1 can promote the translocation of

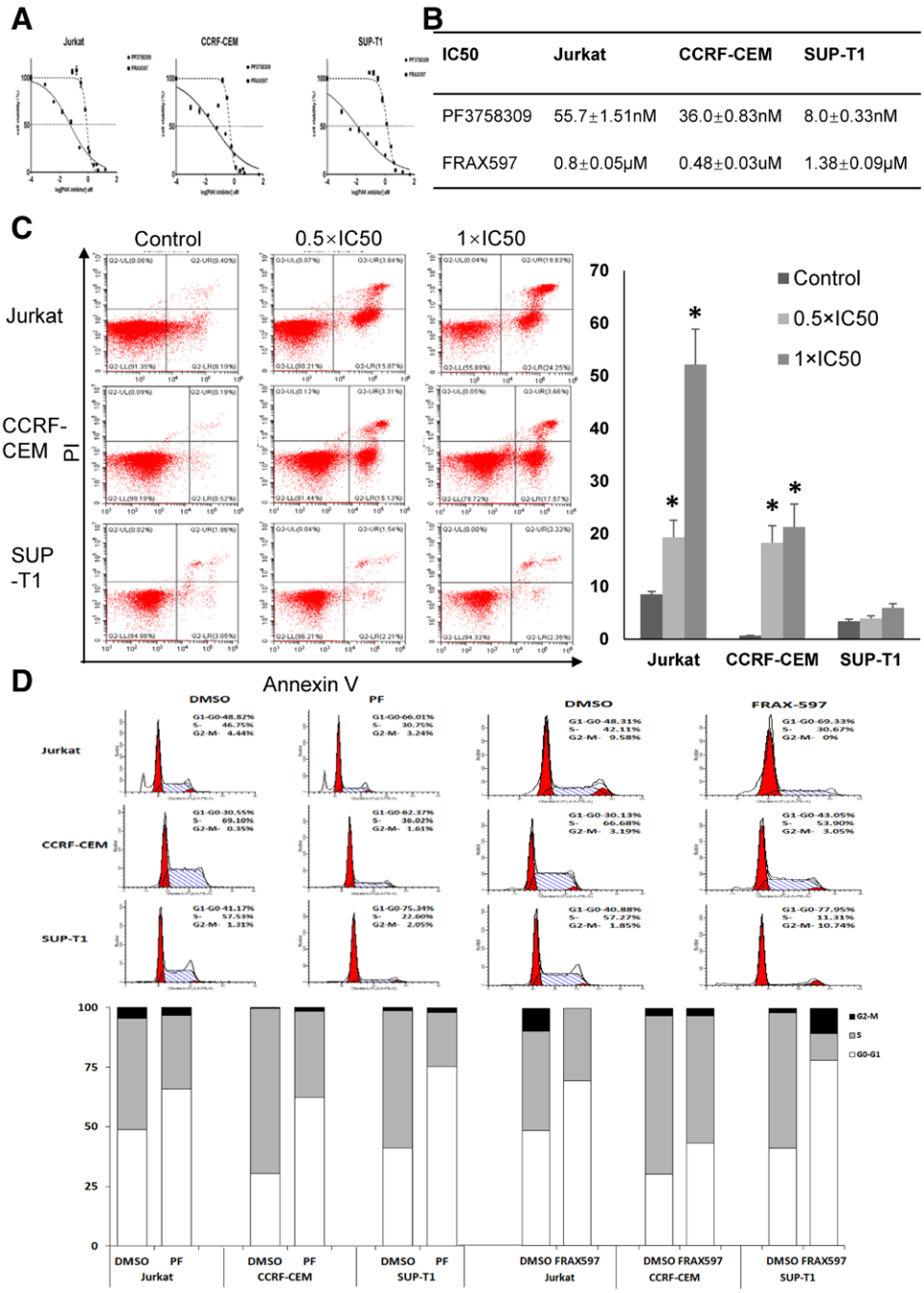

Figure 3. PAKi suppressed T-LBL cells proliferation by regulating the cell cycle. (A) Response curves for PF and FRAX597 intervention in three T-LBL cell lines; (B) IC_{50} values of PF and FRAX597 in three T-LBL cell lines; (C) The apoptosis analysis of three T-LBL cell lines treated with two PF doses for 24 h. The histogram shows the percentage (%) of apoptotic cells. (D) T-LBL cell lines exposed to PF for 24 h at the indicated concentrations were analyzed by flow cytometry for cell cycle changes. Stacked bar graphs indicate the percentage of cells in the G0-1, S, and G2/M phases. *P < .05. IC_{50} = 50% inhibitory concentrations, PAKi = P21-activated kinase inhibitors, PF = PF3758309, T-LBL = T-cell lymphoblastic lymphoma.

the NF-κB p65 subunit into the nucleus to function as a transcription factor.[31] As a result, PAK inhibition may be a viable option for NF-κB-targeted cancer therapy. The current study found that PAKi could suppress proliferation of T-LBL cells in vivo and in vitro, which might be caused by the downregulation of cyclin D1, NF-κB and reduced phosphorylation levels of PAK1/2 protein. However, the mechanism of PAKs' biological function in T-LBL is quite intricate. PAK can also influence the proliferation and survival of tumor cells through feedback control of the nuclear transcription process via downstream pathways. These downstream pathways include TCR/NF-κB, STAT5, ERK, β-catenin, and Raf/BAD.[32,33] In this study, KEGG analysis showed that cell adhesion-related genes were downregulated in T-LBL cells treated with PAKi in vitro. It is worthwhile to further investigate the regulatory mechanism of PAKi on cell adhesion pathways. Together, these findings indicate that PAKi might bring therapeutic benefits to T-LBL patients.

Currently, single-agent PAKi and the combination of PAKi and conventional chemotherapy or other targeted agents have shown potential therapeutic value for various malignancies in preclinical studies.[14,34] For instance, PAKi inhibited growth and promoted apoptosis of leukemia cells.[22,35] However, certain adverse events were observed in patients treated with PAKi, including neutropenia and gastrointestinal reactions, which

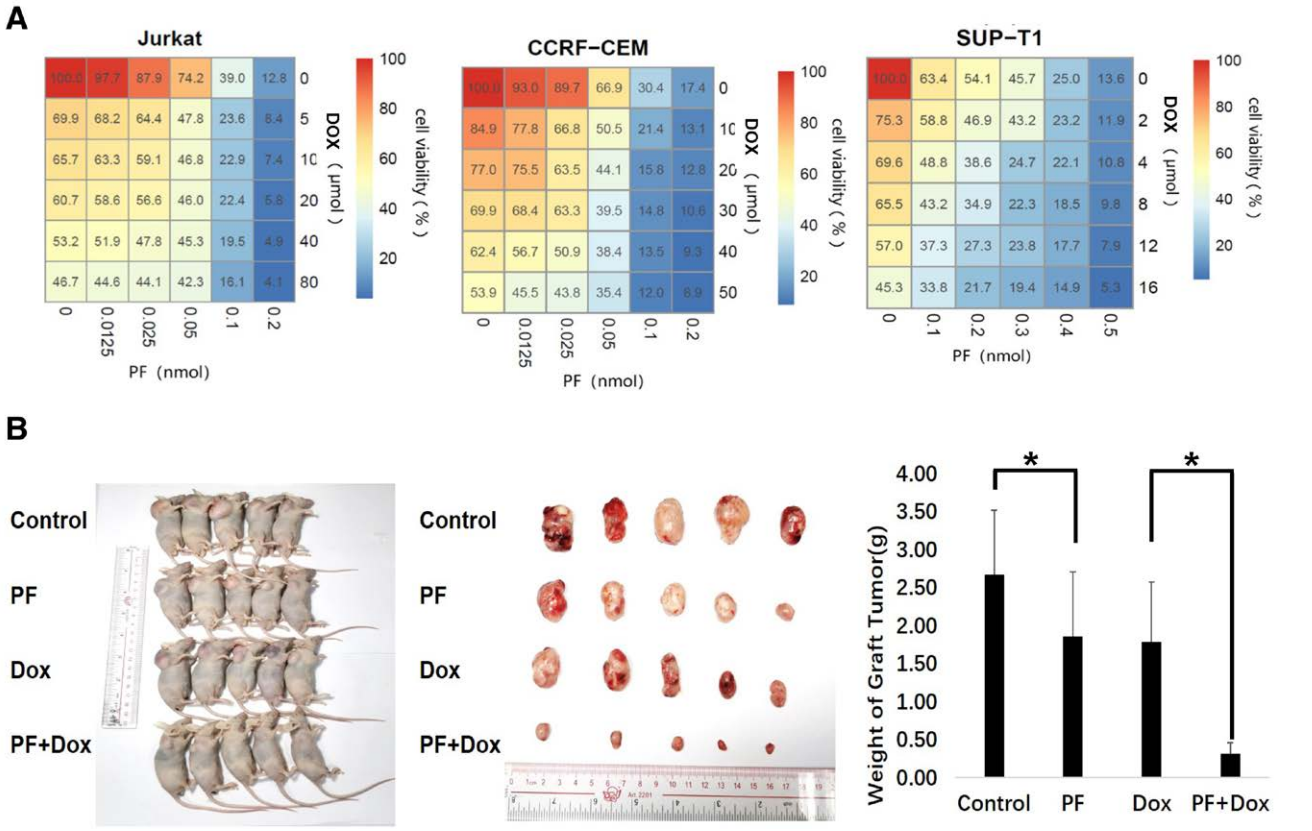

Figure 4. PF enhanced the chemosensitivity of DOX in T-LBL in vitro and in vivo. (A) A heatmap representing the cell viability after 48 h treatment with various concentrations of PF and DOX in three T-LBL cell lines. Red and blue colors represent high and low cell viability, respectively; (B) SUP-T1 cells were injected subcutaneously into athymic nude mice. Tumors were dissected after the treatment with PF/DOX for 14 d. The weight of the transplanted tumors was recorded and compared. *$P < .05$. DOX = doxorubicin, PF = PF3758309, T-LBL = T-cell lymphoblastic lymphoma.

hindered further clinical investigation of PAKi.[36] Moreover, the combination of PAKi and chemotherapy synergistically inhibited cancer cell proliferation and tumor growth in pancreatic cancer.[37,38] Simultaneous inhibition of PAK and other kinases also displayed an enhanced anti-tumor, as reported in the xenograft models of breast cancer and lung cancer.[39–42] In addition, PAKi could improve the therapeutic efficacy of PD-1 antibodies and CAR-T immunotherapy via enhancing T-cell infiltration and reprograming the vascular microenvironment.[43,44] These findings suggest that PAKi might be therapeutically used to overcome drug resistance in cancer therapy. Our study also observed the synergistic effects of PAKi and DOX in T-LBL in vivo and in vitro.

In summary, the present results demonstrate the expression patterns of PAK family members in T-LBL and the association between PAK expression and early relapse of T-LBL. Furthermore, PAKi suppressed proliferation, induced G_0/G_1 cell cycle phase arrest and enhanced the chemosensitivity of DOX in vitro and in vivo. These anti-tumor activities might be induced by the deregulation of PAK phosphorylation, cyclin D1 and NF-κB. Together, the above results suggest that PAK might be a potential therapeutic target for T-LBL patients.

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AUTHOR CONTRIBUTIONS

Q.C.: conceptualization; project administration; funding acquisition; resources; supervision; writing - review and editing. X.T. and X.C.: conceptualization; methodology; investigation; data curation; writing - review & editing. N.S., and Y.F.: methodology; investigation; data curation; writing - original draft. X.C., Z.X., H.H., Y.X., and P.L.: collection of clinical samples; data curation. All authors gave final approval to the manuscript and consent to publish the data.

REFERENCES

[1] Cortelazzo S, Ponzoni M, Ferreri AJ, Hoelzer D. Lymphoblastic lymphoma. *Crit Rev Oncol Hematol* 2011;79(3):330–343.
[2] Sweetenham JW. Treatment of lymphoblastic lymphoma in adults. *Oncology (Williston Park)* 2009;23(12):1015–1020.
[3] Thomas DA, O'Brien S, Cortes J, et al. Outcome with the hyper-CVAD regimens in lymphoblastic lymphoma. *Blood* 2004;104(6):1624–1630.
[4] Huguet F, Leguay T, Raffoux E, et al. Pediatric-inspired therapy in adults with Philadelphia chromosome–negative acute lymphoblastic leukemia: the GRAALL-2003 study. *J Clin Oncol* 2009;27(6):911–918.
[5] Aljurf M, Zaidi SZA. Chemotherapy and hematopoietic stem cell transplantation for adult T-cell. *Biol Blood Marrow Transplant* 2005;11(10):739–754.
[6] Portell CA, Sweetenham JW. Adult lymphoblastic lymphoma. *Cancer J* 2012;18(5):432–438.

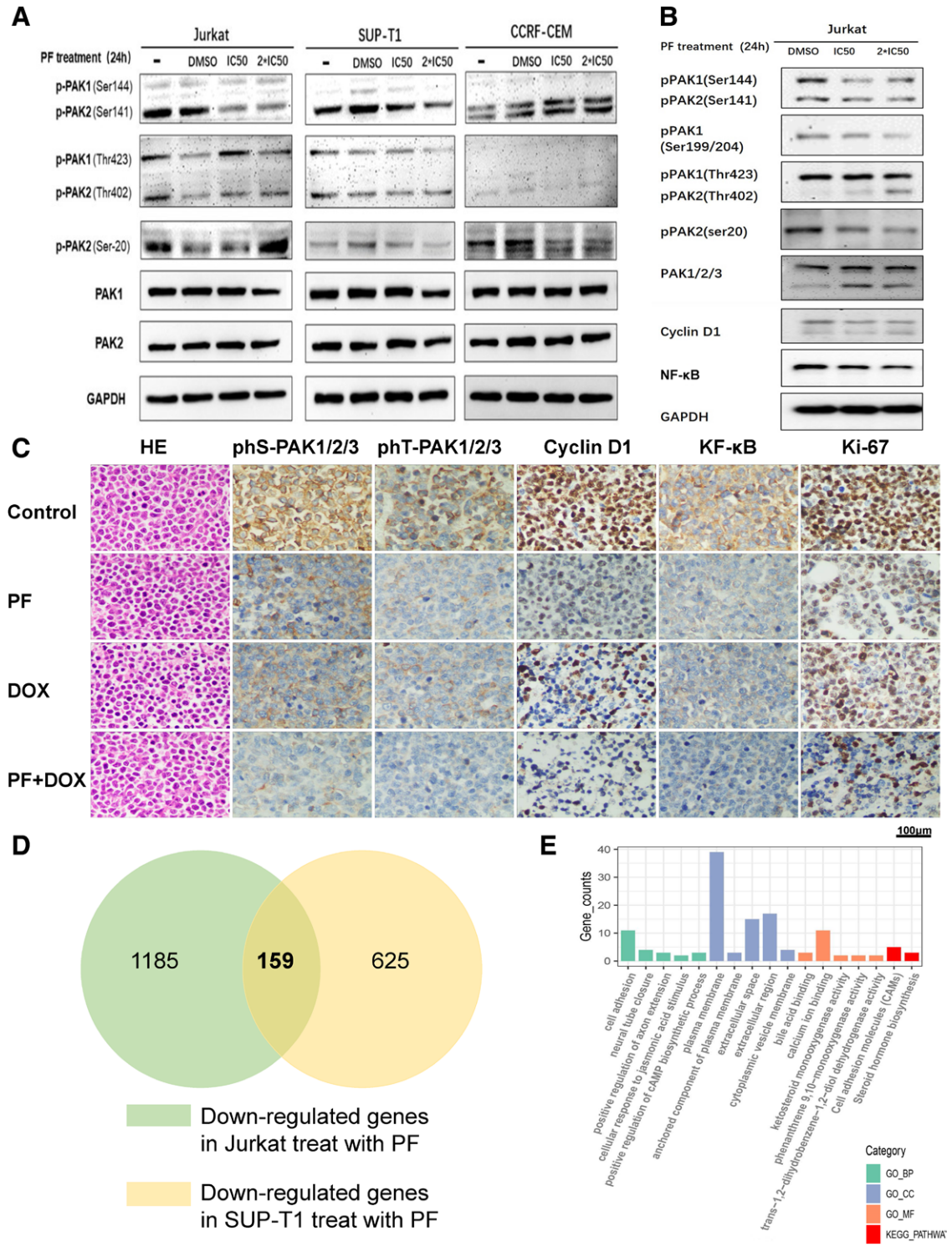

Figure 5. Targets and pathways in T-LBL cells treated with PAKi. (A) Western blotting analysis of total and phosphorylated PAK in T-LBL cells treated with PF at 2 concentrations (IC_{50}, 2 × IC_{50}) for 24 h; (B) Western blot indicated that phosphorylated PAK1/2, cyclin D1 and NF-κB proteins were downregulated by PF in Jurkat cells; (C) The protein levels of phosphorylated PAK1/2/3, Cyclin D1, NF-κB, and ki-67 were tested by immunohistochemical staining in xenograft tumor samples treated after PF/DOX. (D) Venn diagrams showing the intersection between differentially downregulated genes in Jurkat and SUP-T1 after treatment with PF via RNA sequencing analysis; (E) Cell adhesion and other pathways suppressed by PAKi were identified by GO and KEGG enrichment analysis of 159 downregulated genes in both Jurkat and SUP-T1 cells. DOX = doxorubicin, GO = Gene Ontology, IC_{50} = 50% inhibitory concentrations, KEGG = Kyoto Encyclopedia of Genes and Genomes, PF = PF3758309, phS-PAK1/2/3 = anti-PAK1 + PAK2 + PAK3 (phospho S144 + S141 + S154), phT-PAK1/2/3 = anti-PAK1 + PAK2 + PAK3.

[7] Mullighan CG. Mutations of NOTCH1, FBXW7, and prognosis in T-lineage acute lymphoblastic leukemia. *Haematologica* 2009;94(10):1338–1340.
[8] Kindler T, Cornejo MG, Scholl C, et al. K-RasG12D-induced T-cell lymphoblastic lymphoma/leukemias harbor Notch1 mutations and are sensitive to gamma-secretase inhibitors. *Blood* 2008;112(8):3373–3382.
[9] Narayanan S, Shami PJ. Treatment of acute lymphoblastic leukemia in adults. *Crit Rev Oncol Hematol* 2012;81(1):94–102.
[10] Tian XP, Huang WJ, Huang HQ, et al. Prognostic and predictive value of a microRNA signature in adults with T-cell lymphoblastic lymphoma. *Leukemia* 2019;33(10):2454–2465.

[11] Tian XP, Xie D, Huang WJ, et al. A gene-expression-based signature predicts survival in adults with T-cell lymphoblastic lymphoma: a multicenter study. *Leukemia* 2020;34(9):2392–2404.
[12] Tian XP, Su N, Wang L, et al. A CpG methylation classifier to predict relapse in adults with T-Cell lymphoblastic lymphoma. *Clin Cancer Res* 2020;26(14):3760–3770.
[13] Vadlamudi RK, Kumar R. P21-activated kinases in human cancer. *Cancer Metastasis Rev* 2003;22(4):385–393.
[14] Liu H, Liu K, Dong Z. The role of p21-activated kinases in cancer and beyond: where are we heading? *Front Cell Dev Biol* 2021;9:641381.
[15] Qian D, Chen K, Deng H, et al. MicroRNA-374b suppresses proliferation and promotes apoptosis in T-cell lymphoblastic lymphoma by repressing AKT1 and Wnt-16. *Clin Cancer Res* 2015;21(21):4881–4891.
[16] Chen X, Xie R, Gu P, et al. Long noncoding RNA LBCS inhibits self-renewal and chemoresistance of bladder cancer stem cells through epigenetic silencing of SOX2. *Clin Cancer Res* 2019;25(4):1389–1403.
[17] Tian XP, Cai J, Ma SY, et al. BRD2 induces drug resistance through activation of the RasGRP1/Ras/ERK signaling pathway in adult T-cell lymphoblastic lymphoma. *Cancer Commun (Lond)* 2020;40(6):245–259.
[18] Chen J, Bardes EE, Aronow BJ, Jegga AG. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic Acids Res* 2009;37(Web Server issue):W305–W311.
[19] Foucquier J, Guedj M. Analysis of drug combinations: current methodological landscape. *Pharmacol Res Perspect* 2015;3(3):e00149.
[20] Cokol-Cakmak M, Bakan F, Cetiner S, Cokol M. Diagonal method to measure synergy among any number of drugs. *J Vis Exp* 2018;(136):e57713.
[21] Radu M, Semenova G, Kosoff R, Chernoff J. PAK signalling during the development and progression of cancer. *Nat Rev Cancer* 2014;14(1):13–25.
[22] Chung EY, Mai Y, Shah UA, et al. PAK kinase inhibition has therapeutic activity in novel preclinical models of adult T-cell leukemia/lymphoma. *Clin Cancer Res* 2019;25(12):3589–3601.
[23] Pandolfi A, Stanley RF, Yu Y, et al. PAK1 is a therapeutic target in acute myeloid leukemia and myelodysplastic syndrome. *Blood* 2015;126(9):1118–1127.
[24] Quan L, Cheng Z, Dai Y, Jiao Y, Shi J, Fu L. Prognostic significance of PAK family kinases in acute myeloid leukemia. *Cancer Gene Ther* 2020;27(1–2):30–37.
[25] Liu W, Liu Y, Liu H, et al. Tumor suppressive function of p21-activated Kinase 6 in hepatocellular carcinoma. *J Biol Chem* 2015;290(47):28489–28501.
[26] Jiang Y, Liu W, Li T, et al. Prognostic and predictive value of p21-activated Kinase 6 associated support vector machine classifier in gastric cancer treated by 5-fluorouracil/oxaliplatin chemotherapy. *EBioMedicine* 2017;22:78–88.
[27] Parekh P, Rao KV. Overexpression of cyclin D1 is associated with elevated levels of MAP kinases, Akt and Pak1 during diethylnitrosamine-induced progressive liver carcinogenesis. *Cell Biol Int* 2007;31(1):35–43.
[28] Li Y, Wang D, Zhang H, et al. P21-activated kinase 4 regulates the cyclin-dependent kinase inhibitor p57(kip2) in human breast cancer. *Anat Rec (Hoboken)* 2013;296(10):1561–1567.
[29] Dammann K, Khare V, Gasche C. Tracing PAKs from GI inflammation to cancer. *Gut* 2014;63(7):1173–1184.
[30] Li LH, Wu GY, Lu YZ, et al. p21-activated protein kinase 1 induces the invasion of gastric cancer cells through c-Jun NH2-terminal kinase-mediated activation of matrix metalloproteinase-2. *Oncol Rep* 2017;38(1):193–200.
[31] Friedland JC, Lakins JN, Kazanietz MG, Chernoff J, Boettiger D, Weaver VM. alpha6beta4 integrin activates Rac-dependent p21-activated kinase 1 to drive NF-kappaB-dependent resistance to apoptosis in 3D mammary acini. *J Cell Sci* 2007;120(Pt 20):3700–3712.
[32] Kumar R, Li DQ. PAKs in human cancer progression: from inception to cancer therapeutic to future oncobiology. *Adv Cancer Res* 2016;130:137–209.
[33] Reddy PN, Radu M, Xu K, et al. p21-activated kinase 2 regulates HSPC cytoskeleton, migration, and homing via CDC42 activation and interaction with beta-Pix. *Blood* 2016;127(16):1967–1975.
[34] Kumar R, Gururaj AE, Barnes CJ. p21-activated kinases in cancer. *Nat Rev Cancer* 2006;6(6):459–471.
[35] Flis S, Bratek E, Chojnacki T, Piskorek M, Skorski T. Simultaneous inhibition of BCR-ABL1 tyrosine kinase and PAK1/2 serine/threonine kinase exerts synergistic effect against chronic myeloid leukemia cells. *Cancers* 2019;11(10):1544.
[36] Rudolph J, Crawford JJ, Hoeflich KP, Wang W. Inhibitors of p21-activated kinases (PAKs). *J Med Chem* 2015;58(1):111–129.
[37] Yeo D, He H, Patel O, Lowy AM, Baldwin GS, Nikfarjam M. FRAX597, a PAK1 inhibitor, synergistically reduces pancreatic cancer growth when combined with gemcitabine. *BMC Cancer* 2016;16:24.
[38] Wang J, Zhu Y, Chen J, et al. Identification of a novel PAK1 inhibitor to treat pancreatic cancer. *Acta Pharm Sin B* 2020;10(4):603–614.
[39] Korobeynikov V, Borakove M, Feng Y, et al. Combined inhibition of Aurora A and p21-activated kinase 1 as a new treatment strategy in breast cancer. *Breast Cancer Res Treat* 2019;177(2):369–382.
[40] Arias-Romero LE, Villamar-Cruz O, Huang M, Hoeflich KP, Chernoff J. Pak1 kinase links ErbB2 to beta-catenin in transformation of breast epithelial cells. *Cancer Res* 2013;73(12):3671–3682.
[41] Ito M, Codony-Servat C, Codony-Servat J, et al. Targeting PKCiota-PAK1 signaling pathways in EGFR and KRAS mutant adenocarcinoma and lung squamous cell carcinoma. *Cell Commun Signal* 2019;17(1):137.
[42] Ito M, Codony-Servat C, Karachaliou N, Rosell R. Targeting PKCiota-PAK1 in EGFR-mutation positive non-small cell lung cancer. *Transl Lung Cancer Res* 2019;8(5):667–673.
[43] Abril-Rodriguez G, Torrejon DY, Liu W, et al. PAK4 inhibition improves PD-1 blockade immunotherapy. *Nat Cancer* 2020;1(1):46–58.
[44] Ma W, Wang Y, Zhang R, et al. Targeting PAK4 to reprogram the vascular microenvironment and improve CAR-T immunotherapy for glioblastoma. *Nat Cancer* 2020;2(1):83–97.