INTRODUCTION

Pancreatic cancer is a malignant disease, and the death rate is almost equal to the incidence of the disease. Currently, pancreatic cancer is the fourth leading cause of cancer-related death worldwide.\(^1\)

Although significant progress has been made in the diagnosis and treatment of the disease, the 5-year survival rate change little and remained at about 6%.\(^2\) Thus, there is an urgent need for identification of novel treatment targets and a better understanding of the molecular basis of the disease.

The homeodomain-interacting protein kinase (HIPK) family contains four members: HIPK1-3 are structurally similar, while HIPK4

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The homeodomain-interacting protein kinase (HIPK) family contains four members: HIPK1-3 are structurally similar, while HIPK4
is not structurally related to the others outwith the catalytic domain. HIPK1-3 were first identified as Nkx1.2-interacting proteins in yeast two-hybrid screening. Approximately 90% of the amino acid sequences that make up their kinase domains are conserved across HIPK1-3, and their noncatalytic region is also conserved. HIPK family members are expressed in dynamic temporal and spatial patterns, suggesting their diverse and important roles during development. In disease models, HIPKs mediate key signalling pathways that regulate the response to various stress signals, including DNA damage, reactive oxygen species and hypoxia. HIPK2 is the best-studied member of the HIPK family and is involved in the regulation of differentiation, proliferation and apoptosis of cells. In cancer, HIPK2 is generally considered to be a potential tumour suppressor as it can promote apoptosis by phosphorylating tumour suppressor protein p53, leading to expression of proapoptotic genes. HIPK2 also induces apoptosis by modulating molecules independently of p53, such as phosphorylation-dependent degradation of antiapoptotic transcriptional co-repressor CtBP. Due to the structural similarities between HIPK1 and HIPK2, these two proteins share some redundant activities, and HIPK1 also regulates apoptosis via interacting with nuclear proteins. HIPK3 has been reported to regulate the pathogenesis of type 2 diabetes. To date, little is known about the roles of HIPK4 except for its capacity to phosphorylate p53 at Ser9. These reports demonstrate that HIPK proteins might have important roles in cancer and are promising anticancer targets. However, the expression status of HIPKs in pancreatic cancer and their indicative roles in prognosis have seldom been discussed. Thus, there is a need to uncover the role of HIPKs in pancreatic cancer and provide the possible molecular mechanism in the hope of discovering novel treatment targets for pancreatic cancer.

Ninety years ago, Otto Warburg published a body of work linking metabolism and cancer, demonstrating that tumour cells rapidly use glucose and convert the majority of it into lactate, which is known as the Warburg effect. Through aerobic glycolysis, tumour cells utilize glucose to meet the demand of uncontrolled proliferation. Lactate produced by aerobic glycolysis creates an acidic environment that favours metastasis of cancer cells, as the extracellular matrix becomes destabilized under acidic conditions. Activated oncogenes or loss of tumour suppressors could alter metabolism and induce aerobic glycolysis. In pancreatic cancer, mutation of KRAS oncogene induces metabolism reprogramming via induction of enhanced aerobic glycolysis. Tumour suppressor gene TP53 could induce the expression of the TP53-induced glycolysis regulatory phosphatase (TIGAR), which is a regulator of aerobic glycolysis. The cMyc oncogene is a master regulator that controls many aspects of cancer cell malignancy, and it is usually overexpressed in cancer cells that have been transformed into a more malignant phenotype. cMyc also induces enhanced aerobic glycolysis in cancer cells by induction of key glycolytic genes like glucose transporter 1 (GLUT1), hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA). Thus, cMyc is considered to be a gatekeeper that balances uncontrolled proliferation and enhanced aerobic glycolysis. In this regard, an understanding of cellular metabolic derangements in pancreatic cancer and uncovering the molecular mechanism underlying the derangement could lead to novel therapeutic approaches.

In the present study, we examined the indicative roles of HIPKs in pancreatic cancer prognosis and demonstrated that HIPK2 predicted better prognosis. In vitro studies demonstrated that overexpression of HIPK2 inhibited pancreatic cancer proliferation and aerobic glycolysis. Mechanistic studies demonstrated that HIPK2 decreased cMyc protein stability via post-translational phosphorylation of cMyc via extracellular signal-regulated kinase (ERK). This work might provide novel predictive and treatment targets for pancreatic cancer.

## MATERIALS AND METHODS

### Cell culture

Human pancreatic cancer cell lines PANC-1, MIA PaCa-2, BxPC-3, SW1990 and Capan-1 were obtained from the American Type Culture Collection (ATCC) and cultured according to standard protocols provided by ATCC. Human epithelial cell line HPDE was kindly provided by Professor Min Li at the University of Oklahoma Health Sciences Center.

### Real-time polymerase chain reaction

TRizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from pancreatic cancer cells. To obtain cDNA, TaKaRa PrimeScript RT Reagent Kit (TaKaRa Bio, Shiga, Japan) was used for reverse transcription. The expression status of specific genes and control β-actin gene was determined by quantitative real-time polymerase chain reaction (PCR) using an ABI 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences are listed in Table 1.

### Western blotting

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (150 mmol/L NaCl, 1% NP-40, 50 mmol/L Tris/HCl, pH 7.4) and provided by Professor Min Li at the University of Oklahoma Health Sciences Center.

| Primer sequences used in the text |
|-----------------------------------|
| HIPK2 forward 5'-TATTGGTGAGTTGATAGCTGG-3' |
| HIPK2 reverse 5'-GGTCGGGTGATGGCTGAG-3' |
| cMyc forward 5'-AGAGTCGGATCACCTTCTGTAAG-3' |
| cMyc reverse 5'-ACATTCTCCGTGTCGGAGAC-3' |
| GLUT1 forward 5'-GACGACGCTACCTTGGATGCTC-3' |
| GLUT1 reverse 5'-CATACTGGAAGCACATGCCAC-3' |
| HK2 forward 5'-GATTGGTGTAACCTTCTCAGA-3' |
| HK2 reverse 5'-TTTGTCTGGCTTGCTGAG-3' |
| LDHA forward 5'-GATTCAGTTGCTGCTTATAG-3' |
| LDHA reverse 5'-CACCTCATAAGCACTTCTACCC-3' |
| β-actin forward 5'-CCAACGGCAGAGATGACCCA-3' |
| β-actin reverse 5'-ATACGATGCCAGTGTCAG-3' |
HCl, pH 8.0 and 10% glycerol) containing protease and phosphatase inhibitors (Selleck, Shanghai, China). Cell debris was removed by centrifugation at 10,000 g for 20 minutes at 4°C. Protein concentrations of the whole cell lysate were measured using Thermo Pierce® BCA Protein Assay Kit (Rockford, IL, USA). Equal total amounts of proteins were subjected to SDS-PAGE separation and transferred to polyvinylidene difluoride membranes. Antibodies against ERK1/2 and phospho-ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA, USA). cMyc and phospho-S62-cMyc antibodies were obtained from Abcam (Cambridge, MA, USA). HIPK2, GLUT1, HK2, LDHA and β-actin antibodies were produced by Proteintech (Rosemont, IL, USA). FLAG antibody was manufactured by Sigma-Aldrich (St. Louis, MO, USA).

2.4 | Plasmid and lentivirus production

HIPK2, cMyc and wild-type ERK2 was cloned into pCDH-CMV-MCS-EF1-Puro plasmid. Kinase dead mutant of ERK2K52R was generated using a site-directed mutagenesis kit and cloned into pCDH-CMV-MCS-EF1-Puro vector. Addgene’s pLKO.1 TRC cloning vector was used to generate shRNA-expressing constructs. 21bp targets against HIPK2 were CCCACAGCACACACGTCAAAT and GTTCTTGTTGGCCGTTATA, respectively. Lentivirus was produced by confection of targeted gene-expressing vector with psPAX2 and pMD2.G into HEK293T cells in a ratio of 4:3:1.

2.5 | CCK-8 and colony formation assay

Cell variability was determined using Cell Counting Kit-8 reagents (Dojindo, Japan). Cells were seeded in 96-well plates (3 × 10^3 cells per well). 10 μL CCK-8 solution was added to each well at 0, 24, 48 and 72 hours, and the plates were incubated at 37°C in 5% CO₂ for 1 hour. The absorbance of each sample was measured at a wavelength of 450 nm using a microplate reader. Colony formation was performed as described previously.²⁵ Pancreatic cancer cells (n = 500) were seeded in six-well plates and cultivated for 10-14 days. The cells were fixed with 4% paraformaldehyde followed by staining with 1% crystal violet staining. The colonies were counted subsequently.

2.6 | cMyc protein stability assessment

Pancreatic cancer cells were treated with cycloheximide (CHX; 100 μg/mL) and harvested for the indicated times, followed by Western blotting and quantification of the blot bands.

2.7 | Glycolysis measurement

Glucose utilization and glycolysis were measured using glucose uptake assay, lactate production assay and Seahorse extracellular flux analyzer. Glucose Uptake Colorimetric Assay Kit (Biovision, MilPitas, CA, USA) and Lactate Colorimetric Assay Kit (Biovision) were used to examine glucose intake and lactate generation by pancreatic cancer cells. Seahorse extracellular flux analyzer was used to measure glycolysis by examining extracellular acidification rate (ECAR) and to measure mitochondrial respiration by measuring oxygen consumption rate (OCR). In brief, pancreatic cancer cells were seeded into XF96-well cell culture plates and incubated overnight. Subsequently, the cells were washed in pre-warmed XF assay media (for OCR measurement, XF assay media was supplemented with 10 mmol/L glucose, 1 mmol/L pyruvate, 2mmol/L l-glutamine and adjusted at pH 7.4). Cells were then incubated in 175 μL/well of XF assay media at 37°C, in a non-CO₂ incubator for 1 hour. During incubation, 25 μL of 80 mmol/L glucose, 9 μmol/L oligomycin, 1 mol/L 2-deoxyglucose (for ECAR measurement) and 25 μL of 10 μmol/L oligomycin, 9 μmol/L FCCP, 10 μmol/L rotenone, 10 μmol/L antimycin A (for OCR measurement) in XF assay media was loaded into the injection ports of the XFe-96 sensor cartridge. During the experiment, the instrument injected these inhibitors into the wells at a given time point, while ECAR/OCR was measured continuously. ECAR and OCR measurements were normalized by protein content (Sulphorhodamine B assay). Data sets were analysed by XFe-96 software, using one-way ANOVA and Student’s t test calculations. All experiments were performed in triplicate.

2.8 | Immunohistochemistry

Expression of HIPK2 in pancreatic cancer patients’ samples was assessed by immunohistochemistry. Paraffin sections were incubated for 1 hour at 70°C, deparaffinized in xylene and rehydrated in graded ethanol. The slides were neutralized with 3% H₂O₂ for 30 minutes. The antigen retrieval was processed with citrate buffer (pH 6.0) in an incubator at 95°C. After antigen retrieval, the slides were incubated for 1 hour at 70°C, deparaffinized in xylene and rehydrated in graded ethanol. The slides were neutralized with 3% H₂O₂ for 30 minutes. The antigen retrieval was processed with citrate buffer (pH 6.0) in an incubator at 95°C. After antigen retrieval, the slides were incubated with primary and secondary antibodies. HIPK2 antibody (ab28507; Abcam) was used at a dilution of 1:50. The sections were stained with 3,3-diaminobenzidine, terminated in PBS and counterstained with haematoxylin.

2.9 | Tissue specimens

The clinical tissue samples used in this study were histopathologically and clinically diagnosed at Fudan University Shanghai Cancer Center (FUSCC). Prior patient consent and approval from the Institutional Research Ethics Committee were obtained.

2.10 | The Cancer Genome Atlas dataset analysis

TCGA-PAAD on RNA expression (Level 3) of pancreatic cancer patients in terms of RNA-seq by Expectation-Maximization was downloaded from the Cancer Genomics Brower of the University of California, Santa Cruz (UCSC) (https://genome-cancer.ucsc.edu/). In total, 177 primary pancreatic cancer samples from patients with detailed expression data were chosen from the updated TCGA database according to the parameters mentioned. Detailed demographics of these patients were characterized by the TCGA consortium.
2.11 | Statistical analyses

Statistical analyses were performed using SPSS version 17.0 (IBM Corp., Armonk, NY, USA) using independent Student’s t test (two-tailed) or one-way analysis of variance. Logistic regression was used to determine the correlation between HIPK2, GLUT1, HK2 and LDHA expression level and clinicopathological characteristics in the TCGA cohorts. Statistical significance was based on two-sided \( P < 0.05. \)

3 | RESULTS

3.1 | Decreased HIPK2 expression indicates worse prognosis of pancreatic cancer

To investigate the indicative roles of HIPK expression in pancreatic cancer prognosis, we assessed expression of HIPK1-4 in TCGA database. We demonstrated that patients with lower HIPK2 expression exhibited worse prognosis, and lower HIPK2 correlated with worse overall survival (OS) and disease-free survival (DFS) (Figure 1A,B). Expression of HIPK1, HIPK3 and HIPK4 had no indicative roles in prognosis of pancreatic cancer (Figure S1A-F). The clinicopathological characteristics of the TCGA-included pancreatic cancer patients are listed in Table S1. We validated the roles of HIPK2 in pancreatic cancer OS in a FUSCC cohort using immunohistochemical staining. The scoring of HIPK2 staining was indicated as Low HIPK2 and High HIPK2 (Figure S2). Consistent with the TCGA cohort, patients with lower HIPK2 expression displayed shorter OS (Figure 1C). Subsequently, we assessed expression of HIPK2 in pancreatic cancer and adjacent para-tumour tissues. Quantitative PCR demonstrated that expression of HIPK2 was higher in para-tumour samples than in tumour samples (Figure 1D). Immunohistochemical staining demonstrated that HIPK2 expression was significantly lower in tumour than para-tumour samples (Figure 1E,F). Collectively, these results demonstrated that HIPK2 might play negative roles in pancreatic cancer prognosis prediction and function as a tumour suppressor.

3.2 | HIPK2 negatively regulates proliferation of pancreatic cancer cells

To validate the roles of HIPK2 in vitro, we measured expression of HIPK2 in pancreatic cancer cell lines. HIPK2 expression was lower in PANC-1 and SW1990 cells, but it was higher in normal epithelial cell line HPDE (Figure 2A). Thus, we overexpressed FLAG-tagged HIPK2 in PANC-1 and SW1990 cells (Figure 2B,C). We performed a CCK8 assay to confirm the roles of HIPK2 in pancreatic cancer cell variability. The results demonstrated that overexpression inhibited variability of PANC-1 and SW1990 cells (Figure 2D). Moreover, silencing of HIPK2 expression in HPDE cells by lentivirus mediated transfection method also demonstrated that silencing HIPK2 expression could increase the cell variability of HPDE cells (Figure S3A-C). Colony formation assay suggested that introduction of HIPK2 into PANC-1 and MIA PaCa-2 cells inhibited colony formation capacity (Figure 2E,F). Analysis of apoptosis revealed that overexpression of HIPK2 in pancreatic cancer cells promoted apoptosis (Figure 2G,H). These results show that HIPK2 might act as a negative regulator of proliferation in pancreatic cancer cells.

3.3 | HIPK2 functions as a negative regulator of aerobic glycolysis in pancreatic cancer

It is well known that enhanced aerobic glycolysis promotes uncontrolled proliferation of cancer cells; thus, we investigated whether HIPK2 negatively regulated aerobic glycolysis. In the process of aerobic glycolysis, cancer cells utilize glucose to produce lactate, instead of the mitochondrial respiration pathway. Glycolysis and mitochondrial respiration can be measured by Seahorse extracellular flux analyzer, with ECAR to reflect aerobic glycolysis and OCR to indicate mitochondrial respiration. HIPK2 overexpression decreased glycolysis and glycolytic capacity, which was reflected by ECAR measurement (Figure 3A,B). Mitochondrial respiration was impaired in the process of aerobic glycolysis, resulting in a reduction of oxygen consumption that was reflected by OCR measurement. We observed that HIPK2 overexpression enhanced OCR, suggesting its positive roles in mitochondrial respiration (Figure 3C,D). Collectively, these results demonstrate that HIPK2 is a negative regulator of aerobic glycolysis.

3.4 | HIPK2 downregulates cMyc and cMyc-targeted glycolytic genes

In HIPK2-overexpressing PANC-1 and SW1990 cells, we measured the changes in cMyc levels. Quantitative PCR and Western blotting showed a significant reduction in cMyc protein levels, while the mRNA levels decreased only slightly (Figure 4A,B). cMyc-targeted glycolytic genes including GLUT1, HK2 and LDHA had decreased mRNA and protein levels in HIPK2-overexpressing PANC-1 and SW1990 cells (Figure 4C,D). In pancreatic cancer patients, expression of HIPK2 negatively correlated with expression of GLUT1, HK2 and LDHA (Figure 4E-G). Thus, HIPK2 regulates aerobic glycolysis via cMyc and cMyc transcription of glycolytic genes.

3.5 | HIPK2 regulates aerobic glycolysis via cMyc

HIPK2 induces a decrease in cMyc protein levels, suggesting that cMyc mediates HIPK2-induced aerobic glycolytic regulation. We rescued cMyc expression in HIPK2-overexpressing cells (Figure 5A). By assessing glucose uptake and lactate generation, we demonstrated that cMyc<sup>WT</sup> attenuated the decrease in glucose uptake caused by HIPK2 overexpression in PANC-1 and SW1990 cells (Figure 5B). Cancer cells utilize glucose to produce lactate via glycolysis, which can be measured by lactate
FIGURE 1  HIPK2 indicates better prognosis and is downregulated in pancreatic cancer. Kaplan-Meier analysis using TCGA pancreatic adenocarcinoma cohort demonstrated that patients with lower HIPK2 expression had better OS than those with lower HIPK2 expression (A). DFS analysis with TCGA pancreatic adenocarcinoma cohort demonstrated that HIPK2 levels could serve as a predictor of tumour recurrence, and patients with higher HIPK2 expression had better DFS (B). By using FUSCC pancreatic cancer cohort and immunohistochemical staining, we demonstrated that higher HIPK2 expression indicated better prognosis (C). Quantitative PCR analysis demonstrated that HIPK2 expression was higher in adjacent tumour samples than the tumour samples (D). Immunohistochemical staining confirmed that HIPK2 expression was higher in adjacent normal tumour samples (E and F)
FIGURE 2  HIPK2 negatively regulates proliferation of pancreatic cancer cells. HIPK2 expression was lower in PANC-1 and SW1990 cells and exhibited highest expression in HPDE cells (A). FLAG-tagged HIPK2 was overexpressed in PANC-1 and SW1990 cells, and the overexpressing efficacy was confirmed by quantitative PCR and Western blot analysis (B, C). HIPK2 introduction into PANC-1 and SW1990 cells inhibited variability, as demonstrated by CCK-8 assay (D). In HIPK2 stable overexpression cell lines, the colony formation capacity of PANC-1 and SW1990 cells was inhibited (E, F). HIPK2 overexpression increased apoptosis of PANC-1 and SW1990 cells (G, H).
production. In line with the glucose uptake assay, cMyc mitigated the decrease in lactate levels caused by HIPK2 introduction in PANC-1 and SW1990 cells (Figure 5C). Consistent with these results, cMyc\(^{WT}\) increased expression of glycolytic genes including GLUT1, HK2 and LDHA in HIPK2-overexpressing cells (Figure 5D).

### 3.6 HIPK2 regulates cMyc stability via suppressing ERK activation

As observed above, we demonstrated that HIPK2 decreased cMyc protein levels more than mRNA levels, suggesting that HIPK2 regulates cMyc post-translationally. By assessing the half-life of cMyc,
FIGURE 4 HIPK2 downregulated cMyc and cMyc-targeted glycolytic genes. HIPK2 overexpression decreased cMyc protein levels in PANC-1 and SW1990 cells (A). HIPK2 had a small impact on cMyc mRNA levels (B). Real-time PCR demonstrated that HIPK2 decreased cMyc-targeted glycolytic genes, including GLUT1, HK2 and LDHA (C). Introduction of HIPK2 into PANC-1 and SW1990 cells decreased GLUT1, HK2 and LDHA protein levels (D). In TCGA cohort of pancreatic cancer patients, we observed a negative and significant correlation between HIPK2 with GLUT1, HK2 and LDHA, respectively (E-G)
we demonstrated that HIPK2 overexpression decreased the protein stability of cMyc in PANC-1 and SW1990 cells (Figure 6A,B). We measured changes in ERK1/2 activation in HIPK2-overexpressing cells, and introduction of HIPK2 attenuated activation of ERK1/2 (Figure 6C). ERK phosphorylated Ser62 at cMyc and stabilized it; thus, we measured changes in Ser62-phosphorylated cMyc. In HIPK2 overexpressing cells, by immunoprecipitating cMyc wand subsequent Western blot analysis with pS62-cMyc antibody, we observed a decrease in phosphor-S62 cMyc, suggesting HIPK2 could regulated cMyc stability via ERK-mediated phosphorylation (Figure 6D). To confirm this, we overexpressed ERK2 in HIPK2-overexpressing cells and observed an increase in cMyc protein levels, but the dominant negative form of ERK2K52R could not rescue the decrease in cMyc caused by HIPK2 (Figure 6E).

4 | DISCUSSION

Even though significant progress has been made in the diagnosis, surgery and chemotherapy of pancreatic cancer, there has been no satisfactory improvement in 5-year survival of the disease. Therefore, discovery of novel treatment targets and uncovering the underlying molecular mechanism have become increasingly urgent. In the present study, we indicated that HIPK2 serves a marker for predicting better prognosis of pancreatic cancer. Mechanistic studies have suggested that HIPK2 negatively regulates proliferation and aerobic glycolysis in pancreatic cancer via the ERK/cMyc axis.

The best-characterized tumour-suppressive role of HIPK2 is its modification of p53 post-translational phosphorylation and involvement in p53-induced apoptosis. Due to its impact on p53-mediated apoptosis regulation, HIPK2 is considered to be a tumour suppressor.26 In pancreatic cancer, genetic mutation of TP53 is a high frequency mutation, but as a negative regulator of p53, the roles of HIPK2 have seldom been reported.27 In our present study, we reported HIPK2 to be a tumour suppressor, which is consistent with reports in other types of cancer. One reason that accounts for poor prognosis of pancreatic cancer is its intrinsic resistance to chemotherapy and radiotherapy, which could induce the apoptotic process.28 Thus, discovery of molecular targets that could be used to reverse the treatment resistance of pancreatic cancer might help...
improve prognosis of pancreatic cancer. HIPK2 can be activated by several forms of genotoxic damage, such as UV radiation, ionizing radiation or antitumour drugs like cisplatin, leading to expression of proapoptotic genes. Therefore, decreased HIPK2 expression in pancreatic cancer might play certain roles in resistance to chemotherapy and radiotherapy. Further investigations to demonstrate the role of decreased HIPK2 in pancreatic cancer chemotherapy and radiotherapy resistance are needed.

**FIGURE 6** HIPK2 regulated cMyc stability via suppressing ERK activation. HIPK2 regulated cMyc protein rather than mRNA levels, suggesting that HIPK2 regulated cMyc protein stability. The half-life of endogenous cMyc in control or HIPK2-overexpressing cells was calculated by CHX chase assay, and HIPK2 decreased stability of cMyc in PANC-1 and SW1990 cells (A, B). In HIPK2-overexpressing PANC-1 and SW1990 cells, we observed a reduction in activation status of ERK1/2 (C). In HIPK2 overexpressing cells, we immunoprecipitated endogenous cMyc and subsequently examined the phosphorylated levels of cMyc at Ser62 site. HIPK2 decreased phosphorylated cMyc at Ser62, which positively regulated cMyc stability (D). ERK2 rescued the decrease in cMyc protein levels caused by HIPK2, while the dominant negative mutant of ERK2 (ERK2K52R) had no such effect, suggesting that HIPK2 mediated cMyc stability via ERK activation (E).
Oncogenic mutations in KRAS have been observed in >90% of pancreatic cancer patients. However, attempts to target KRAS are futile and KRAS is considered to be an undruggable target. Therefore, targeting KRAS downstream signalling pathways might provide an alternative.\textsuperscript{32,33} KRAS mutation could lead to constitutive activation of the ERK signalling pathway, which regulates cancer proliferation, metastasis, aberrant cancer cell metabolism and drug resistance.\textsuperscript{34,35} In the present study, we demonstrated that HIPK2 suppressed the activation status of ERK1/2, making it a suitable target for targeting KRAS/ERK activation in pancreatic cancer. Our and other previous studies have demonstrated that ERK activation leads to enhanced cMyc protein stability and protein levels via post-translational phosphorylation and ubiquitination.\textsuperscript{25,36,37} In pancreatic and other types of cancer, cMyc is a master regulator of many aberrancies and is a promising drug target.\textsuperscript{38,39} Some compounds like JQ-1 that target cMyc have been proven to be successful for the treatment of cancer.\textsuperscript{40,41} In the present study, we demonstrated that HIPK2 suppressed cMyc protein stability, which reinforced the concept that HIPK2 might be used as a novel target for the treatment of pancreatic cancer. Another function of cMyc is regulation of metastasis, as it can confer a selective advantage on cancer cells by promoting proliferation, survival, differentiation blockade, genomic instability and angiogenesis; all of which may indirectly contribute to metastasis.\textsuperscript{42,43} There is mounting evidence to indicate that cMyc directly controls cancer invasion, migration and metastasis by inducing expression of downstream metastatic genes. For example, cMyc is reported to regulate epithelial-mesenchymal transition by promoting transforming growth factor β-induced Snail expression.\textsuperscript{44} As a negative regulator of cMyc, HIPK2 is reported to regulate metastasis in many types of cancer, such as lung adenocarcinoma and gastric cancer, but the role of HIPK2 in pancreatic cancer metastasis has seldom been discussed.\textsuperscript{45,46} Therefore, shedding light on the role of HIPK2 in pancreatic cancer metastasis might provide novel strategies for treating pancreatic cancer.

To date, attempts to develop agents to target genetic aberrations to kill cancer cells have been futile. In this regard, an understanding of other targets might provide novel therapeutic targets. Cancer cells are different from normal cells in their proliferation rate and the fine-tuned machinery for utilization of nutrients like glucose. For normal cells, mitochondrial respiration was utilized to provide cells with nutrients to support cell division. Uncontrolled proliferation is prevented because mammalian cells do not normally take up nutrients from their environment unless stimulated to do so by growth factors. To meet the demands for uncontrolled proliferation caused by oncogenic mutations, cancer cells shifted their metabolic pattern from mitochondrial respiration to aerobic glycolysis to survive under hostile conditions characterized by limited oxygen and nutrient supply. Enhanced aerobic glycolysis has emerged as a new target for pancreatic cancer and understanding the underlying molecular mechanism will open new opportunities.\textsuperscript{47-49} cMyc and hypoxia-inducible factor (HIF)1α are reported to be key regulators of aerobic glycolysis, which control the expression of important genes that govern aerobic glycolysis.\textsuperscript{50} Previous studies have demonstrated that HIPK2 suppresses HIF1α mRNA and protein levels in normoxic or hypoxic conditions, resulting in changes in chemotherapy resistance and tumour growth.\textsuperscript{51,52} Whether HIPK2 regulates aerobic glycolysis has seldom been reported before. Thus, it is necessary to examine the role of HIPK2 in HIF1α-induced aerobic glycolysis in the future. The present study demonstrated that HIPK2 regulates aerobic glycolysis via cMyc. As reported previously, cMyc also regulates serine and glutamine metabolism; therefore, it is natural to hypothesize that HIPK2 regulates metabolic reprogramming more than aerobic glycolysis does.\textsuperscript{53,54} In this study, we demonstrated that HIPK2 regulated cMyc stability via post-translational modifications including phosphorylation. As HIPK2 is also a serine/threonine kinase, and cMyc protein stability can be regulated by serine and threonine phosphorylation, there is the possibility that HIPK2 interacts with and phosphorylates cMyc, which causes cMyc destabilization.\textsuperscript{55} The possibility that HIPK2 interacts with cMyc is that they share the same interacting partners such as mitogen-activated protein kinase kinase kinase 1 (MEKK1) and FBW7 (F-Box And WD Repeat Domain Containing 7).\textsuperscript{56,57} Therefore, further investigations to uncover the role of HIPK2 in cMyc post-translational regulation might help the discovery of new phenomena and targets by utilizing HIPK2.

Even though there is mounting evidence that HIPK2 is a tumour suppressor that modulates cell growth, apoptosis, proliferation and development, recent studies have suggested that HIPK2 has positive roles in tumour progression. For example, HIPK2 is a downstream target of NRF2 (nuclear factor, erythroid 2 like 2), which is an important regulator of intracellular redox homeostasis and oncogenes in cancer. Besides, HIPK2 is required for robust NRF2 responsiveness in cancer cells and in vivo.\textsuperscript{58} HIPK2 also controls the levels of Notch1, a well-established factor with dual roles in cancer, that can act as both tumour suppressor and oncogenic factor, depending on the context.\textsuperscript{59,60} These reports add strength to the notion that HIPK2 possesses context-dependent roles in cancer.

In conclusion, we demonstrated that decreased HIPK2 expression is an unfavourable marker for pancreatic cancer OS. Subsequent
in vitro studies demonstrated that HIPK2 regulated proliferation and aerobic glycolysis in pancreatic cancer cells. In-depth mechanistic studies suggested that HIPK2 attenuated activation of ERK and resulted in destabilization of cMyc (Figure 7). Collectively, the present study uncovered novel predictive and treatment targets of pancreatic cancer.

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CONFLICT OF INTEREST

All authors state that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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