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TWIST1 regulates HK2 ubiquitination degradation to promote pancreatic cancer invasion and metastasis

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Abstract

Objective TWIST1 is known to promote glycolysis and contribute to pancreatic cancer development; however, its underlying mechanisms remain poorly understood. This study aims to elucidate the molecular mechanisms by which TWIST1 influences aerobic glycolysis in pancreatic ductal adenocarcinoma (PDAC).

Methods The expression levels of TWIST1, MMP9, MT1-MMP, and FDX1 in clinical tissues and cancer cell lines were assessed using quantitative reverse transcription PCR (QRT-PCR). Cell treatments with Elesclomol-Cu and 2-deoxy-glucose (2DG) were conducted. Immunofluorescence staining and immunoprecipitation analyses were performed to investigate the binding relationship between TWIST1 and HK2. Colony formation and Transwell assays were utilized to evaluate the effects of TWIST1 on cell proliferation, migration, and invasion. Western blotting was employed to detect proteins related to cuproptosis and apoptosis, while ubiquitination assays assessed TWIST1's regulation of HK2 ubiquitination.

Results TWIST1 expression was significantly elevated in PDAC tissues, and over-expression of TWIST1 in PDAC cells enhanced colony formation and cell proliferation. Notably, HK2 levels were markedly higher in pancreatic cancer tissues compared to adjacent normal tissues. TWIST1 was found to directly bind and interact with HK2, showing co-localization in the cytoplasm of PDAC cells. Furthermore, TWIST1 was shown to stabilize HK2 by inhibiting its ubiquitin-mediated degradation. Knockdown of TWIST1 or HK2 enhanced the inhibitory effects of 2DG on cell migration and invasion. Treatment with Elesclomol-Cu and 2DG significantly reduced the expression of the cuproptosis-related factor FDX1 with no impact on other cell death factors.

Conclusion This study demonstrates that TWIST1 regulates the ubiquitination and degradation of HK2, thereby promoting glycolysis-induced cuproptosis and facilitating pancreatic cancer invasion and metastasis. Understanding the underlying mechanisms of PDAC, including the regulation of key proteins such as HK2 by TWIST1, is crucial for developing more effective treatment strategies. Findings highlight the importance of targeting these molecular pathways, which could lead to improved diagnostic and therapeutic approaches, ultimately enhancing patient outcomes and prognosis.

Keywords Pancreatic cancer, TWIST1, HK2, Reprogrammed glucose metabolism, Ubiquitination

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is characterized by aggressive invasion, high mortality rates, and poor prognosis, making it a leading cause of cancer-related deaths worldwide, with a 5-year survival rate of less than 8% [1–3]. The early stages of PDAC often present no obvious symptoms, and the disease is typically diagnosed at an advanced stage due to the lack of accurate diagnostic methods. As a result, surgical removal is often not a viable option. Understanding the underlying mechanisms of PDAC, including the regulation of key proteins such as HK2 by TWIST1, is crucial for developing more effective treatment strategies. Findings highlight the importance of targeting these molecular pathways, which could lead to improved diagnostic and therapeutic approaches, ultimately enhancing patient outcomes and prognosis.

It has long been recognized that the metabolic characteristics of cancer cells are unique. Normal mammalian cells generate energy by completely oxidizing glucose into carbon dioxide and water. In contrast, to meet their high energy demands during rapid growth, cancer cells preferentially utilize glycolysis over oxidative phosphorylation to produce energy, even in the presence of sufficient oxygen. This phenomenon is referred to as aerobic glycolysis [4]. Glycolysis provides essential energy support for the rapid proliferation and invasion of pancreatic cancer cells [5], however, the mechanism is not clarified.

The reprogramming of metabolic pathways is crucial for pancreatic ductal adenocarcinoma (PDAC) tumorigenesis and may be initiated by mutations in the Kirsten rat sarcoma 2 viral oncogene homolog (KRAS). KRAS mutations can exacerbate the progression of pancreatic cancer by enhancing the glycolytic pathway, as evidenced by the increased activity of key enzymes such as hexokinase 2 (HK2), phosphofructokinase-1 (PFK1), and lactate dehydrogenase A (LDHA) [6, 7]. To get enough energy by glycolysis, key regulators of glycolysis are over-expresed in cancer cells, such as HIF-1α, c-Myc and HK2 [8].

Hexokinase (HK) is a tissue-specific isoenzyme that plays a crucial role in glucose metabolism by phosphorylating glucose to form glucose-6-phosphate (G6P). As an enzyme dedicated to glycolysis, hexokinase exists in four classical subtypes, each encoded by different genes [9]. Among these subtypes, hexokinase 2 (HK2) is significantly involved in the glycolytic processes of various tumors. HK2 possesses two enzyme activity domains: the N-terminal active site and the C-terminal active site [10]. Several previous studies have demonstrated that HK2 is abnormally overexpressed in various tumor tissues and is closely associated with poor patient prognosis in cancers such as hepatocellular carcinoma, breast cancer, colorectal carcinoma, and prostate cancer [11–14].

The implications of TWIST1 and HK2 in tumor biology are significant and multifaceted. TWIST1 is a key regulator of epithelial-mesenchymal transition (EMT), a process that enables cancer cells to acquire migratory and invasive properties. By promoting EMT, TWIST1 facilitates tumor progression and metastasis in various cancers, including breast, lung, and pancreatic cancer [15]. HK2 plays a crucial role in glucose metabolism by catalyzing the first step of glycolysis. Its up-regulation in tumors is associated with the Warburg effect, where cancer cells preferentially utilize glycolysis for energy production, even in the presence of oxygen [16]. This metabolic shift supports rapid cell proliferation and survival in the tumor microenvironment. Both TWIST1 and HK2 have been implicated in promoting cell survival under stress conditions, such as hypoxia or nutrient deprivation. Their over-expression can contribute to resistance against conventional therapies, making tumors more aggressive and difficult to treat [17]. Elevated levels of TWIST1 and HK2 have been associated with poor prognosis in several cancers. Their expression levels may serve as potential biomarkers for diagnosis, prognosis, and treatment response, aiding in the development of personalized therapeutic strategies [15, 18]. Targeting the pathways regulated by TWIST1 and HK2 presents opportunities for developing novel therapeutic interventions. Inhibitors aimed at blocking EMT or metabolic pathways linked to HK2 could enhance the efficacy of existing treatments and mitigate resistance mechanisms [15, 19, 20]. Overall, TWIST1 and HK2 are critical players in tumor biology, influencing cancer progression, metabolism, and treatment outcomes across various malignancies. Understanding their roles can pave the way for innovative therapeutic approaches in cancer management.

It has been reported that reprogramming of glucose metabolism is crucial for the development of pancreatic cancer, with glycolysis regulated by TWIST1 playing a significant role. Preliminary experimental results indicate that TWIST1 may target glycolytic genes, including HK2, GLUT1, PKM2, and ENO1, and regulate aerobic glycolysis by directly interacting with the promoters of these genes. Our study demonstrates that deletion of TWIST1 negatively affects the expression of glycolytic enzymes, while over-expression of TWIST1 positively regulates them. Although the critical role of the interaction between TWIST1 and HK2 in pancreatic ductal adenocarcinoma (PDAC) is evident, the underlying mechanism remains unclear [21].

In this study, we demonstrated that TWIST1 enhances glycolysis in pancreatic ductal adenocarcinoma (PDAC) by promoting HK2 expression, which in turn facilitates the invasion and metastasis of pancreatic cancer.

Materials and methods

Clinical samples

All clinical samples, including human pancreatic cancer tissue and adjacent non-cancerous tissue, were obtained from Shandong Provincial Hospital affiliated with Shandong First Medical University. Written informed consent was obtained from all patients prior to enrollment, and this study was approved by the Research Ethics Committee of Shandong Provincial Hospital. This study included a total of 39 patients, among which there were 20 males and 19 females. There were 24 patients aged 65 and older, and 15 patients under 65.

Cell cultured 2-Deoxy-D-glucose

PDAC cell lines (PL45, MIA-PACA-1, CFPAC-1, and PANC-1) were purchased from ATCC. Cells were treated with Elesclomol-Cu (a potent copper ionophore that promotes copper-dependent cell death through cuproptosis) and 2DG (2-Deoxy-D-glucose, a glucose analog that serves as a competitive inhibitor of glucose metabolism, inhibiting glycolysis by affecting hexokinase).

Lenti-virus construction and transfection

The human TWIST1 open reading frame (ORF) was synthesized and cloned into the pReceiver-Lv241 mammalian expression vector. The shRNA targeting human TWIST1 was cloned into the plenti-shRNA vector. The recombinant lenti-TWIST1 and TWIST1-shRNA vectors were packaged using a lenti-virus packaging system that included pPACK-GAG, pPACK-REV, and pPACK-VSV vectors. The recombinant lentiviral particles were then transfected into 293 T cells using Lipofectamine reagent. Subsequently, the viral supernatant was collected and used to transfect the target cells.

Edu cell proliferation assay

After inoculating the target cells into 96 well plates at the density of 10,000/well, the Edu solution was diluted using DMEM at a ratio of 1:1000 and added into 96 well plate at a dose of 100 μL per well. After two hours, the Edu reagent was eliminated and cells were washed by PBS for 5 min. All cells were fixed using 4% paraformaldehyde and stained by Apollo stain solution. The DNA stain was carried out using 1×Hoechst33342 reaction solution. The fluorescence images were recorded by fluorescence microscope.

Colony formation assay

The cells were seeded on the six well plate with a density of 1000 per well. Ten days later, the cells were fixed with paraformaldehyde and stained with 0.5% crystal violet for 30 min. All the representative images were recorded and cell numbers were counted by two scholars independently.

Immunoprecipitation

Beyotime Biotechnology purchases IP lysis buffers that lyse cells transfected with HA-labeled TWIST1, FLAG-labeled HK2 or vectors. The incubated cells were then added with anti-HA or anti-FLAG antibodies or control homologous IgG conjugate beads overnight at 4 °C. In the next day, the beads were washed using cold lysis buffer for three times. We then added 1×SDS loading buffer into beads solution and boiled it for five minutes and carried out western blot assay to detect the interaction between TWIST1 and HK2.

Transwell assay

Transwell test was adopted. The cells of logarithmic growth phase were digested and collected with trypsin, and the cells were adjusted to a suspension density of 5×10^7 cells /ml for use. Refer to the instructions provided by the Transwell kit. 250 μl cell suspension was taken and added into the upper layer of Transwell chamber (the invasion experiment was to add Matrigel basement membrane matrix gel accompanying the kit to the bottom of Transwell chamber), and 500 μl DMEM medium containing 10%FBS was added into the lower layer of Transwell chamber. Incubated at 37 °C in 5% CO_2 incubator for 24 h. The Transwell chamber was removed, and the remaining medium on the upper part of the chamber and the cells that did not pass through the chamber were carefully wiped with a clean cotton swab. The chamber was placed in 4% paraformaldehyde solution and fixed for 30 min. After the chamber was removed and dried, crystal violet staining was carried out. The number of transmembrane cells in 5 high-power field (×400) of each group was randomly counted by inverted microscope and photographed.

Western blot

Cells were washed using phosphate buffer for three times and lysed using RIPA buffer (Beyotime Biotechnology, P0013B) containing β Mercaptoethanol on ice for five minutes. Next, protein lysate was centrifugated at 4 ℃ for 10 min and the supernatant was collected carefully. All the protein supernatants were mixed with 5×SDS loading buffer (SDS-PAGE protein loading buffer 5×, code: P0015, Beyotime Biotechnology). Before SDS-PAGE electrophoresis, the protein samples were boiled for 5 min. The protein samples were separated using PAGE gel electrophoresis and transferred to Nitrocellulose membranes at constant current condition. The NC membranes were blocked with 5% bovine serum albumin

for 60 min at 26 °C, followed by incubation with primary antibodies which were diluted by 1%BSA overnight at 4℃ conditions.

Immunofluorescent staining

For cell immunofluorescent staining assay, target cells were seeded onto rounded coverslips in 24-well plates and incubated at 37 ℃ for three days in 5% CO_2 condition. For TWIST1 and HK2 staining, cells on coverslips were incubated with TWIST1 or HK2 antibody for 75 min at 26 °C and subsequently treated with Alexa Fluor 594-conjugated or Alexa Fluor 488-conjugated secondary antibodies at 26 °C and under light-free condition for 75 min. The cell nucleus was stained using DAPI (obtained from Sigma) at room temperature for 30 min. All the immunofluorescence images were recorded using fluorescence microscope or laser scanning confocal microscopy.

In vitro ubiquitination assay

Target protein was ubiquitinated using 500 nM hUBA1 (Ubiquitin-like modifier activating enzyme1), 5uM E2, 10uM TAMRA (Carboxy tetramethyl Rhodamine) labelled ubiquitin, At 37 ℃, the magnetic bead suspension was incubated at different times. The samples were separated in 4–12% NuPAGE gel (Invitrogen) under MOPS buffer (Life Technologies) and fluorescent TAMRA treatment, and were recorded by using the ChemiDoc XRS system (Bio-Rad).

Data download and processing

In the R (version 4.3.1) environment, the TCGA plot package was utilized to process the comprehensive pan-cancer transcriptomic and clinical data obtained from the TCGA official website. Subsequently, we extracted transcriptomic and clinical data specifically pertaining to uterine corpus endometrial carcinoma (UCEC) patients from the TCGA plot package for subsequent analysis. To explore the enrichment of immune response-related gene sets within the UCEC samples, we employed the single-sample gene set enrichment analysis (ssGSEA) algorithm. ssGSEA is a computational methodology employed to assess gene set enrichment within individual samples, thus providing insights into the activity levels of immune-related genes across diverse samples.

Survival analysis

We conducted survival analysis employing the "survival" and "survminer" packages in R to construct Kaplan–Meier survival curves. Survival differential analysis was carried out using the "survdiff" function, which compared survival time and event status across distinct groups (experimental and control groups). The chi-square statistic was computed, yielding the significance P-value. Subsequently, the "survfit" function was utilized to compute and visualize Kaplan–Meier survival curves, with each curve depicting the survival status for different groups and indicating the corresponding significance P-value. Finally, the "ggsurvplot" function was employed to generate plots depicting survival curves.

Enrichment analysis

We conducted enrichment analysis utilizing the "GSEABase," "ClusterProfiler," and "org.Hs.eg.db" packages, in conjunction with the MetaScape website. The databases utilized for enrichment analysis were derived from the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Enrichment analysis was executed employing the "EnrichGO" function, wherein pathways with a P-value < 0.05 were deemed significantly enriched. Visualization of the results was accomplished using the "ggplot2" and "ggpubr" packages.

Statistical analysis

Statistical collected and analyzed by performed using SPSS 17.0 software and data of measurement were presented as mean ± standard deviation. Student's t test used comparisons between two groups. Besides, one-way ANOVA method was used for comparisons among three or more groups. Values of $P < 0.05$ were considered as statistical significant. All statistical analyses were performed using R, with the corresponding statistical methods set up in the R software. Statistical significance was set at $P < 0.05$. Significance levels were denoted as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

TWIST is highly expressed in pancreatic cancer and may affects patient prognosis through the key enzyme HK2 in sugar metabolism.

To investigate the correlation between TWIST1 and pancreatic cancer, we analyzed the expression of TWIST1 in pancreatic cancer tissues and normal pancreatic tissues using data from the TCGA and GTEx databases. The results indicated that TWIST1 was significantly overexpressed in pancreatic cancer tissues (Fig. 1A). We further examined the expression of TWIST1 across different cell types within pancreatic cancer tissues using a single-cell database (Fig. 1B). Moreover, we found that pancreatic cancer patients with high TWIST1 expression had shorter median overall survival (OS) and disease-free survival (DFS) in the TCGA database (Fig. 1C, D), suggesting that TWIST1 promotes pancreatic cancer proliferation.

Next, in the TCGA-PAAD dataset, we analyzed the correlation between TWIST1 and all protein-coding

genes (Fig. 1E). Based on the descending order of correlation coefficients, we conducted Gene Set Enrichment Analysis (GSEA) on 50 Hallmark pathways (Fig. 1F). The results revealed that these genes were primarily enriched in inflammatory pathways and glucose metabolism pathways. Given the established link between tumor growth and glucose metabolism, we further analyzed the correlation between TWIST1 and the key glycolytic enzyme HK2, uncovering a significant positive correlation between TWIST1 and HK2 expression in pancreatic cancer (Fig. 1G). Additionally, HK2 expression was found to be elevated in pancreatic cancer tissues from both the TCGA and GTEx databases (Fig. 1H), and single-cell analysis showed high HK2 expression in tumor cells within pancreatic cancer tissues (Fig. 1I). Notably, high HK2 expression was negatively correlated with OS and DFS in pancreatic cancer patients (Fig. 1J, K).

In summary, these results suggest that TWIST1 is highly expressed in pancreatic cancer and influences the progression of the disease and patient prognosis by modulating the expression of the key glycolytic enzyme HK2.

TWIST1 was up-regulated in Pancreatic *Cancer* Tissues

We collected pancreatic cancer tissues and adjacent normal tissues to investigate the differential expression of TWIST1 between the two. Western blot and immunohistochemistry (IHC) assays revealed that TWIST1 expression was significantly higher in pancreatic cancer tissues compared to adjacent normal tissues (Fig. 2A, B). These results suggest that TWIST1 may play an important role in regulating pancreatic cancer progression.

TWIST1 affects prognosis of pancreatic *Cancer* patients

In order to explore the main factors affecting the prognosis of pancreatic cancer, we utilized Kaplan–Meier survival analysis for univariate screening. The results indicated that gender, TNM staging, TWIST expression, HK2 expression, tumor location, CA199, and CEA were significantly correlated with the survival of pancreatic cancer patients (Table 1, Fig. 3). Subsequent multivariate regression analysis of the seven suspicious factors identified from the univariate screening showed that the expression of TWIST significantly affects the survival status of patients ($P=0.011$), with low-expression patients having a 0.285-fold probability of death compared to the high-expression group. Additionally, CEA levels can also significantly affect patient survival ($P=0.02$), with normal patients having a 0.333-fold probability of death compared to those with elevated levels. Other factors did not significantly affect the prognosis of pancreatic cancer patients (Table 2).

TWIST1 contributed to proliferation in PDAC cells

We performed western blot assay in several PDAC cell-lines to investigate the expression of TWIST1. As shown in Fig. 4A, the results showed that TWIST1 protein was significantly expressed in PDAC cell lines of PL45, MIA-PACA-1, CFPAL-1 and PANC-1 cells but not in normal human pancreatic duct epithelial cell line of HPDE6-c7. To investigate the impact of TWIST1 on cell proliferation, we constructed TWIST1 knockdown cell lines and TWIST1 over-expression cells using lentiviral transfection (Fig. 4B, C). Edu staining analysis indicated that treatment with shRNA targeting TWIST1 reduced cell proliferation, whereas over-expression of TWIST1 promoted cell proliferation (Fig. 4D). The results of colony formation assays showed that knockdown of TWIST1 led to a decrease in colony numbers compared with the control group, while stable over-expression of TWIST1 in PDAC cells enhanced their colony-forming ability (Fig. 4E).

Effect of TWIST1 on *cancer* cell migration and invasion

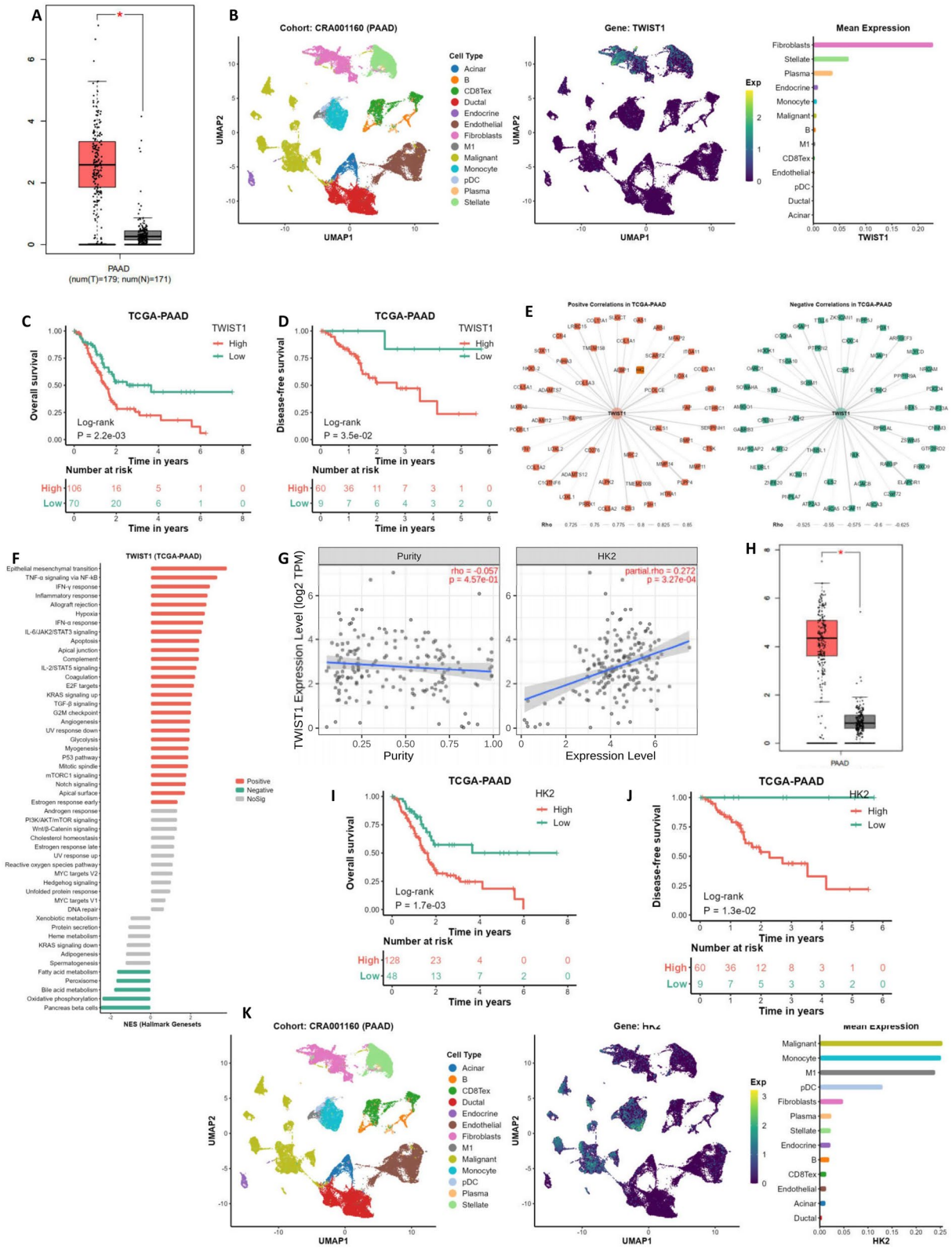
Effects of TWIST1 on the invasion and migration of cancer cells was observed. over-expression of TWIST1 promoted the cell of migration and invasion, and silencing of TWIST1 inhibited that effect (Fig. 5A, $P<0.01$). The scratch assay results showed that TWIST1 knockout affected the process of wound healing (Fig. 5B, $P<0.01$).

TWIST1 directly interact with HK2

We performed immunoprecipitation (IP) experiments and immunofluorescence staining to investigate whether TWIST1 could directly bind to the HK2 protein. The results of the immunofluorescence staining showed that TWIST1 and HK2 co-localize in the cytoplasm

(See figure on next page.)

Fig. 1 Bioinformatics analysis of TWIST in pancreatic cancer. **A**. Differential expression of TWIST1 in tumor tissue and normal tissue in pancreatic cancer TCGA and GTEx databases. **B** Location map of TWIST1 in single-cell sequencing of pancreatic cancer. **C**. K-M survival curve (OS) of TWIST1 low expression patients and high expression patients. **D**. K-M survival curve (DFS) of TWIST1 low expression patients and high expression patients. **E**. Top 50 genes positively or negatively correlated with TWIST1 in TCGA-PAAD. **F**. GSEA enrichment analysis of 50 Hallmark pathways of TWIST1-related proteins. **G**. Correlation test between TWIST1 and HK2 in pancreatic cancer. **H**. Differential expression of HK2 in tumor tissue and normal tissue in pancreatic cancer TCGA and GTEx databases. **I**. K-M survival curve (OS) of HK2 low expression patients and high expression patients. **J**. K-M survival curve (DFS) of HK2 low expression patients and high expression patients. **K**. Location map of HK2 in single-cell sequencing of pancreatic cancer



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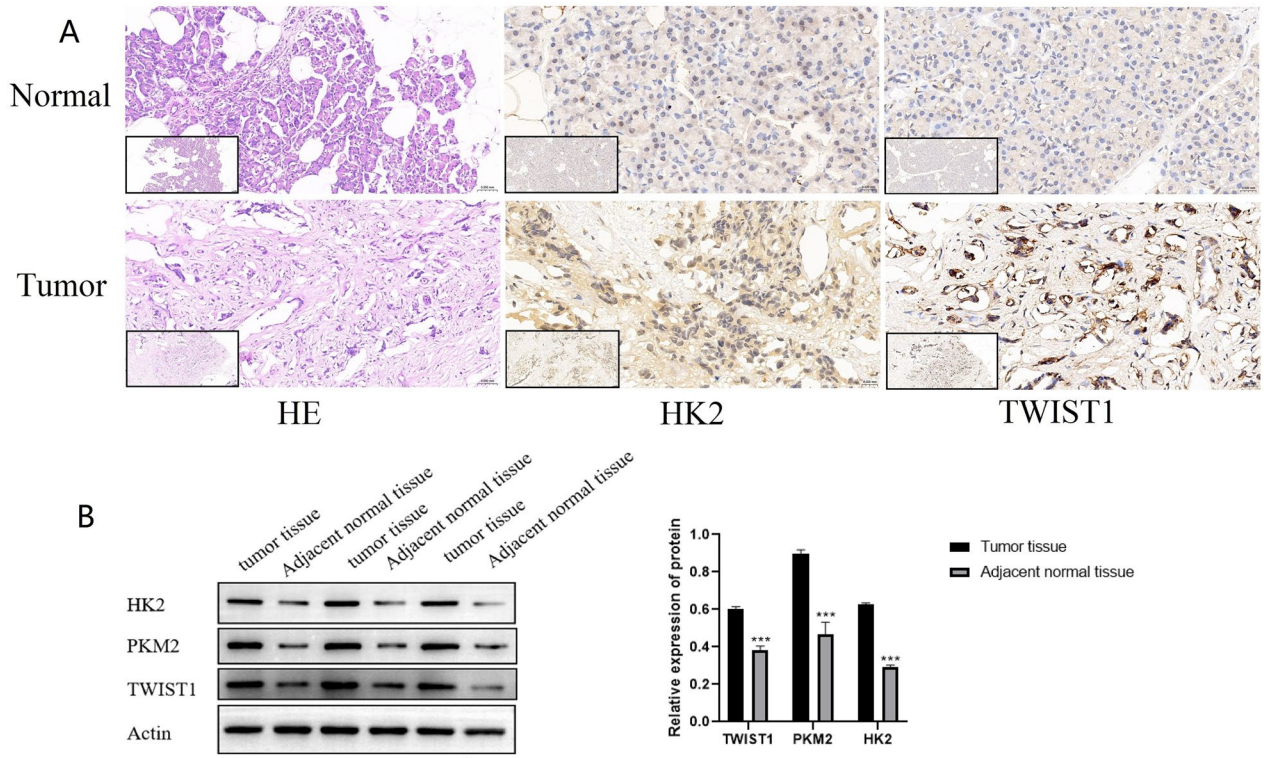


Fig. 2 The expression of TWIST1 and HK2 in pancreatic cancer. **A**. The expression of TWIST1, PKM2 and HK2 in pancreatic cancer tissues in adjacent normal tissue. **B**. The expression of TWIST1, PKM2 and HK2 in pancreatic cancer tissues was significantly higher than that in adjacent normal tissue

of pancreatic cancer cells (Fig. 6 A). Additionally, the IP experiment results demonstrated that TWIST1 can directly bind to HK2 (Fig. 6 B, C). These findings may reveal that TWIST1 is able to directly interact with the HK2 protein.

Effects of HK2 on *cancer* cell migration and invasion

In this article, we conducted a detailed analysis of the sequencing data. In the analysis, we found that TWIST is positively correlated with HK2. Therefore, we doubt whether TWIST can affect cell invasion and metastasis through HK2, so we used 2DG and si-HK2 to conduct experimented. Cells were treated with 2DG (glycolysis inhibitor). The results revealed that 2DG inhibited migration and invasion, OE-TWIST1 reversed 2DG, sh-TWIST1 and si-HK2 promoted the action of 2DG (Fig. 7).

TWIST1 regulates the expression of HK2 and PKM2

It is reported that HK2 acts as key enzyme in glycolytic pathway and catalyzes hexose into hexose-6-phosphate. PKM2 (M2 pyruvate kinase) catalyzes phosphoenolpyruvate (PEP) to pyruvate. Both HK2 and PKM2 play important role in aerobic glycolysis process. In this study, we investigated the effect of TWIST1 on HK2 and PKM2 expression regulation. The results revealed that overexpression of TWIST1 can lead to increased expression of HK2 and PKM2. In coincidence with the above results, we also found that inhibition of TWIST1 caused decrease of HK2 and PKM2 expression A (Fig. 8).

TWIST1 inhibits the ubiquitination degradation of HK2

Ubiquitination degradation pathway is the main protein degradation pathway of HK2. In this study we explored the regulation effect of TWIST1 on HK2 ubiquitination degradation process. The results showed that TWIST1 can stabilize the HK2 function via inhibiting the ubiquitination degradation process of HK2 (Fig. 9).

TWIST1 inhibit the cuproptosis mechanism

The glycolytic pathway often leads to cuproptosis, which is also key to promoting cancer cell metastasis. Here we observed whether TWIST1 promotes cell invasion and migration through the HK2-induced cuproptosis pathway. The expression of cuproptosis related gene FDX1 was detected WB, sh-TWIST1 promoted FDX1 expression, while over-expression inhibited FDX1 expression (Fig. 10A). Elesclomol-Cu (copper pulse) and 2DG treated

Table 1 Univariate analysis of prognostic factors in pancreatic cancer

		OS	Log rank (Mantel-Cox)	P
Sex	Male	9	4.678	0.031*
	Female	18		
Age	≥ 65	14	0.083	0.773
	< 65	10		
TNM	I + II	18	8.144	0.004
	III + IV	6		
TWIST	High	18	20.879	< 0.05*
	Low	6		
HK2	High	18	19.43	< 0.05*
	Low	6		
Pain on the back and loin	Without	7	0.021	0.886
	With	12		
Smoking history	No	11	0.271	0.603
	Yes	12		
Diabetes history	No	11	0.081	0.776
	Yes	12		
Tumor location	Head	7	8.179	0.017*
	Body	9		
	Tail	17		
CA199(U/ml)	Rise	18	4.224	0.04*
	Normal	7		
CEA (ng/ml)	Rise	17	7.061	0.008*
	Normal	7		

cells. WB detected the expression of FDX1, cle-PARP, p-MLKL, GSDMD and GSDME. Elesclomol-Cu and 2DG inhibited FDX1 and Elesclomol-Cu + 2DG, respectively. However, the expression of cle-PARP, p-MLKL, GSDMD and GSDME was not obvious (Fig. 10B, C). The research findings indicate that TWIST1 affects the proliferation, invasion, and metastasis of pancreatic cancer solely by influencing cuproptosis, rather than through other forms of cell death such as ferroptosis and pyroptosis.

Discussion

To meet the needs of proliferation and invasion, cancer cells have modified their energy metabolism, especially for the utilization of glucose. A classical metabolic adaptation of cancer cells is the substitution to aerobic glycolysis as the main source of ATP, which is also known as "Warburg effect" [22, 23]. Glycolysis and oxidative phosphorylation are trading off and taking turns, that is, the increase of glycolysis activity is usually accompanied by the decrease of oxidative phosphorylation level. This metabolic transition is caused by abnormal activation of glycolytic enzymes and is usually reversible [24]. Therefore, in-depth exploration of the driving factors and regulatory mechanism of glycolysis in PDAC will help better understand the pathogenesis of pancreatic cancer.

As a member of the basic helix-loop-helix protein family, TWIST1 is a highly conserved transcription factor that was first found in Drosophila melanogaster [25]. In tumor pathology, TWIST1 is crucial to epithelial-to-mesenchymal transition (EMT), a key mechanism for cancer cells to promote invasion and metastasis [26–29]. It was proved that p53(-/-) pancreatic epithelial cells undergone EMT and expressed high levels of vimentin and the transcriptional regulators including TWIST1, Zeb1 and Zeb2, hinting that p53 inactivation may in turn promote TWIST1 expression [30–32]. Moreover, TWIST1 has also been considered to play a dominant role in initiation, angiogenesis, invasion, stemness, metastasis, and

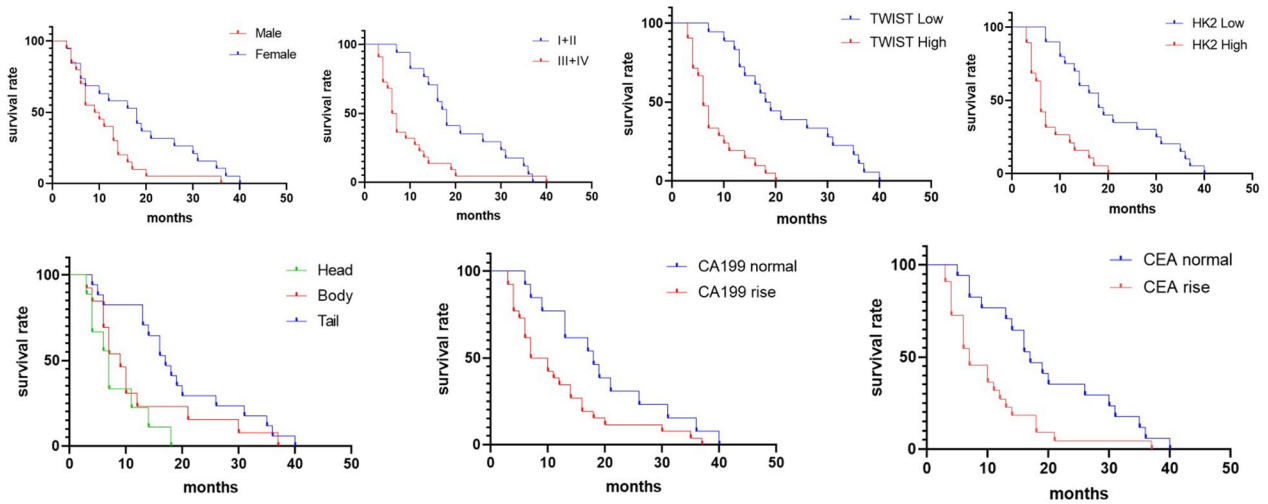


Fig. 3 Kaplan–Meier survival curves for patients with different clinical features

Table 2 Multivariate analysis of prognostic factors in pancreatic cancer

		B	SE	P	OR	OR 95.0% CI	
						Lower	Upper
Sex	Female	0.669	0.444	0.132	1.952	0.818	4.661
	Male	1			1		
TNM	III + IV	−0.650	0.457	0.155	0.522	0.213	1.279
	I + II	1			1		
HK2	Low	−0.818	0.528	0.122	0.441	0.157	1.243
	High	1			1		
TWIST	Low	−1.257	0.492	0.011*	0.285	0.108	0.747
	High	1			1		
Tumor location	Tail	0.784	0.592	0.186	2.189	0.686	6.991
	Body	0.654	0.512	0.201	1.922	0.705	5.241
	Head	1			1		
CA199(U/ml)	Normal	0.311	0.440	0.479	1.365	0.576	3.236
	Rise	1			1		
CEA (ng/ml)	Normal	−1.099	0.473	0.020*	0.333	0.132	0.843
	Rise	1			1		

* means $P < 0.05$,

(See figure on next page.)

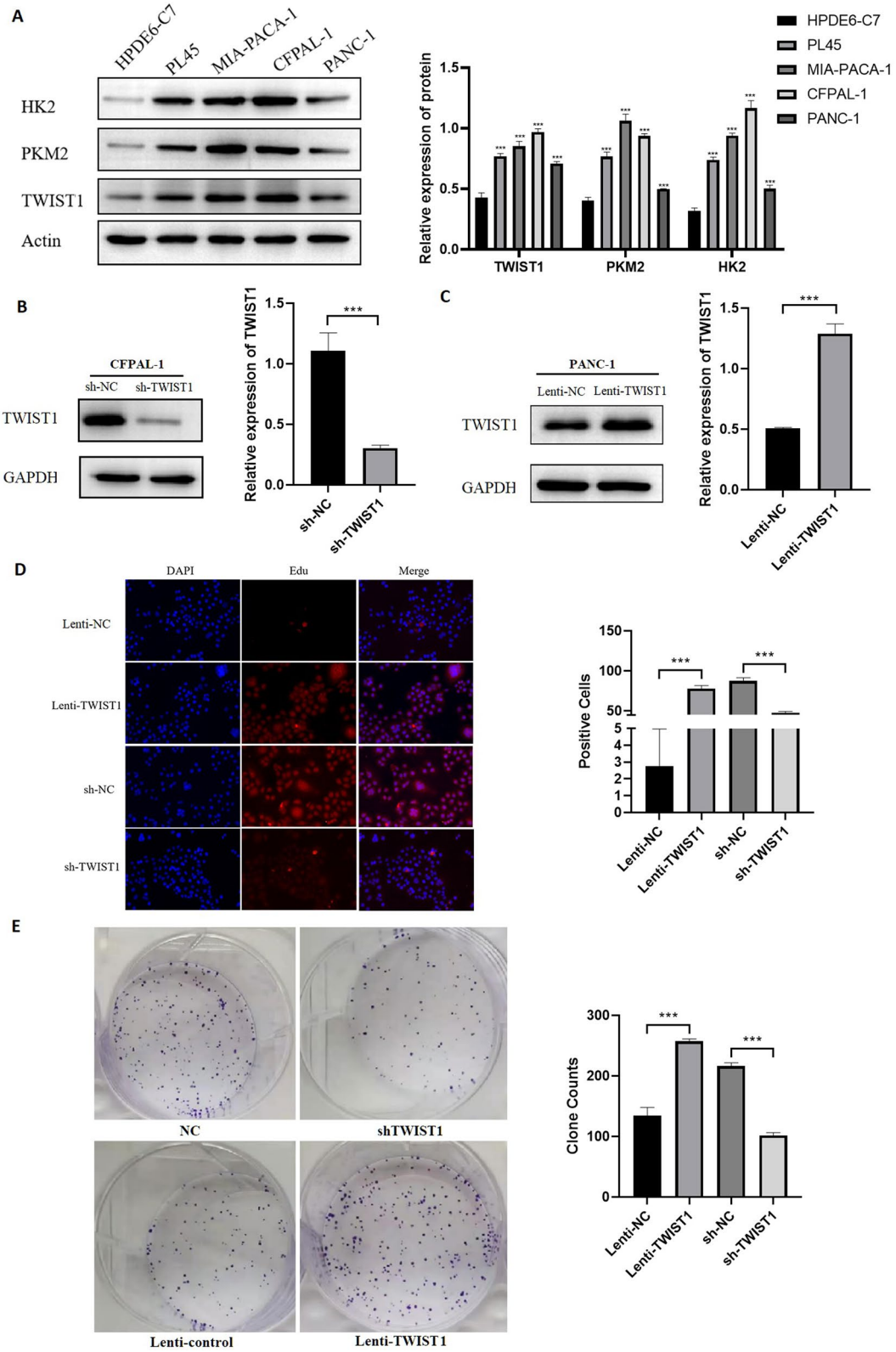
Fig. 4 TWIST1 contributed to proliferation in PDAC cells. **A**. TWIST1, PKM2 and HK2 protein were significantly expressed in PL45, MIA-PACA-1, CFPAL-1 and PANC-1 cells. **B**. Knockdown of TWIST1 in TWISCFPAL-1 cell line. **C**. over-expression of TWIST1 in PANC-1 cell line. **D**. Red fluorescence represented EDU positive, which could be used as a representative of cell proliferation activity. Blue represented the nucleus. The magnification was 200 times. Cell proliferation was reduced by treatment with shRNA against TWIST1 and was promoted by over-expression of TWIST1. **E**. Clone formation number: knockdown TWIST1 inhibited cell cloning, while over-expression played a role in promoting cell cloning

chemo-resistance in a suite of human cancers, such as bladder cancer, breast cancer, cervical cancer and non-small-cell lung cancer [33–37]. TWSIT1 can participate in a variety of physiological and pathological processes by regulating downstream target genes, including AKT2, ARF, etc. [33]. For pancreatic cancer, TWIST1 could promote ubiquitin-mediated proteasomal degradation of HIF-1α and regulate AKT pathways [38]. Through the correlation with EZH2 and Ring 1B, TWSIT1 can promote the development and metastasis of pancreatic cancer under hypoxic conditions [39]. In addition, TWIST1 is also involved in the progress of cisplatin resistance in pancreatic cancer, which is mediated by promoting the expression of GDF15 [40].

A series of researches have proved that TWIST1 could translationally regulate glycolytic genes to promote the Warburg effect in PDAC [41]. Our previous studies suggest that TWIST1 was highly expressed in PDAC tissues too. At the same time, the results of this study shown that TWSIT1 could promote the glucose uptake and lactic acid production of tumor cells, that is, promoted the glycolysis activity of pancreatic cancer cells. Besides, we found that TWIST1 might regulate aerobic glycolysis could by targeting HK2, GLUT1, PKM2 and ENO1, which was crucial for tumor invasion and metastasis. According to previous literature, the boosted glycolysis level wound promotes the proliferation and invasion of tumor cells, which had a negative impact on the prognosis of patients. Then TWIST1 was confirmed closely bound up the glycolysis pathway, which could promote tumor invasion and metastasis.

TWIST is a well-characterized transcription factor that plays a pivotal role in various biological processes, including embryonic development, cell migration, and the regulation of cellular metabolism. Its involvement in the inhibition of HK2 (Hexokinase 2) ubiquitination is particularly intriguing, as it highlights the intersection between transcriptional regulation and post-translational modifications.

As a transcription factor, TWIST can modulate the expression of genes that encode proteins involved in the ubiquitination pathway. This includes deubiquitinating enzymes (DUBs) that specifically target HK2. By enhancing the expression of certain DUBs, TWIST may promote the stabilization of HK2, counteracting the effects of E3 ligases that facilitate its ubiquitination and



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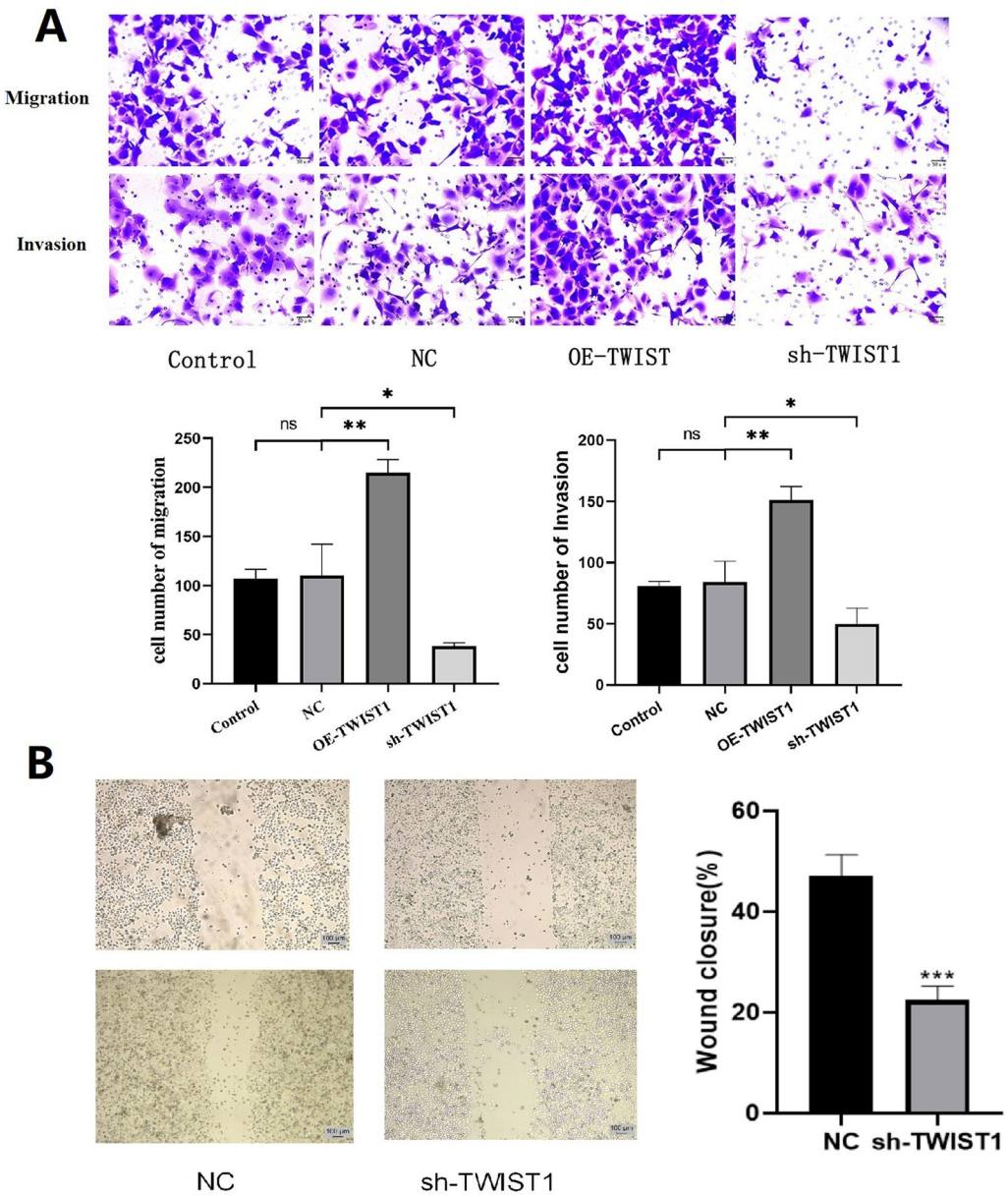

Fig. 5 Effect of TWIST1 on cancer cell migration and invasion Transwell assay detected the migration and the invasion of cancer cell

subsequent degradation [19]. TWIST may also impact the activity of E3 ligases that mediate the ubiquitination of HK2. If TWIST can bind to these ligases or influence their expression, it may reduce the efficiency of HK2 ubiquitination. This competitive inhibition could help maintain HK2 levels, especially under conditions where glucose metabolism is critical for cell survival [42]. TWIST is known to be up-regulated in response to various stressors, such as hypoxia or oxidative stress. Under such conditions, TWIST may exert a protective effect by stabilizing HK2, allowing cells to maintain glycolytic flux and ATP production [42, 43]. This adaptation is particularly important in cancer cells, where TWIST promotes metabolic reprogramming that favors survival and proliferation. TWIST may also interact with other signaling pathways that influence HK2 stability. For example, it could work in concert with pathways involved in cellular survival (such as the PI3K/Akt pathway) to enhance HK2 expression and inhibit its ubiquitination indirectly [18]. TWIST appears to play a multifaceted role in the regulation of HK2 ubiquitination through transcriptional control, competitive inhibition of ubiquitination machinery,

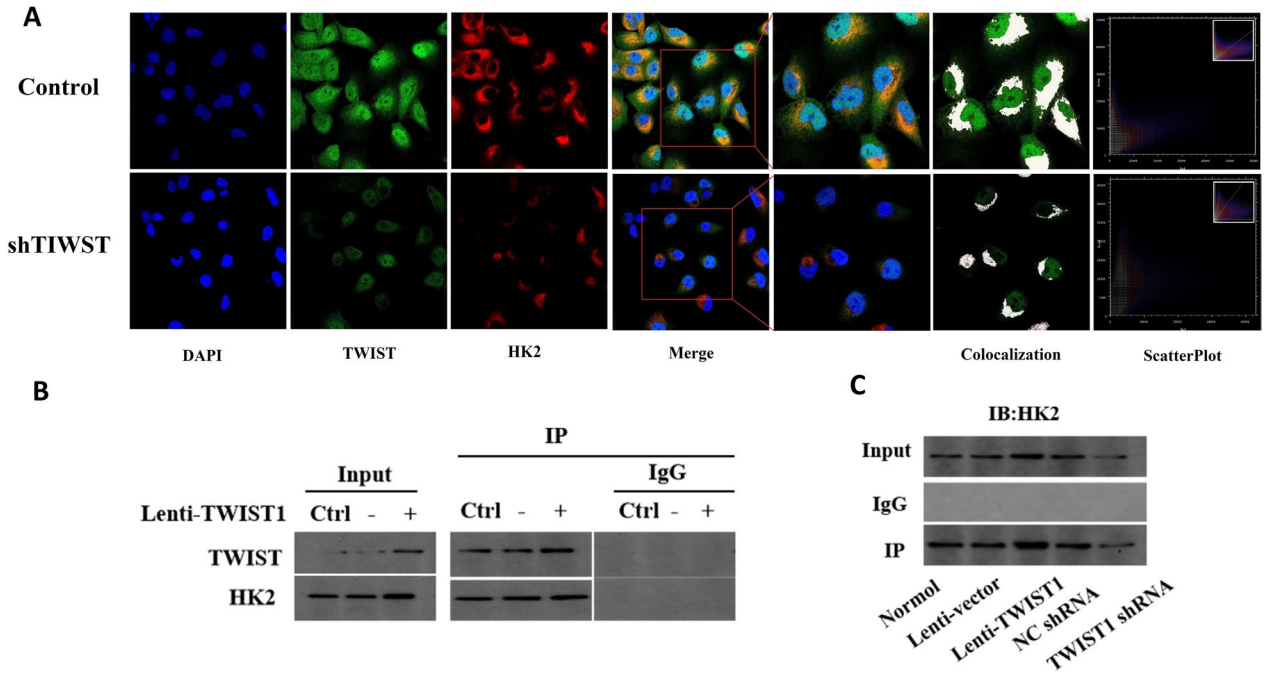

Fig. 6 The interaction between TWIST1 and HK2. **A**. The green fluorescence represents TWIST1, the red fluorescence represents HK2, and the blue protein represents the nucleus. TWIST2 colocalized with HK2 in cytoplasm of PDAC cells. TWIST1 directly bind with HK2-IP assay. **B**.TWIST1 stabilize the HK2 function via inhibiting the ubiquitination degradation process of HK2. **C**. Co-immunoprecipitation analysis of HK2 in different cell lines

and modulation of stress responses [20]. Understanding these interactions in greater depth may provide new insights into the mechanisms by which cells adapt their metabolism in response to environmental challenges, and could have implications for targeting metabolic pathways in cancer therapy.

Through the analysis of the JASPAR database, the author found that TWIST1 could combine with including HK2, ENO1, PKM2, multiple glycolytic genes, which further confirmed that TWIST1 was strongly linked to tumor metabolism [21]. To expound the function of TWIST1 in tumor energy metabolism, we focused on the key gene in glycolysis process, HK2, which is the principal enzyme in glycolysis. HK2 can phosphorylate glucose into glucose-6-phosphate (G6P), which is the speed limiting step of glycolysis.

Consistent with the microarray data and cancer genome atlas dataset previously reported, we found that the expression of HK2 in pancreatic cancer tissue was significantly higher than that in adjacent normal tissue, which indicates that HK2 is crucial to the proliferation of PDAC.

It is identified many times that glucose metabolism reprogramming is critical to the occurrence and development of PDAC, where HK2 extensively participate in. In previous study, it was observed that dysregulation of HK2 was especially essential for the tumorigenesis. Promotion of activity and expression of HK2 has been observed in most types of cancers[10]. Especially, enhanced activity of HK2 is required for the initiation and maintenance of K-Ras-driven cancers, and its inhibition is shown to reduce tumor growth both in vitro and in vivo [44]. There is evidence that HK2 can also be used as a prognostic marker in patients with PDAC.

Besides, chronic inflammation is a key feature of tumor development. It has been determined that the miR-155/miR-143/HK2 axis may be a pathway that links the inflammatory response with metabolic reprogramming in cancer cells. In terms of mechanism, miR-155 mainly promotes the glycolysis of tumor cells by up regulating HK2, and then exerts its cancer promoting function [45]. Additionally, It has been indicated that aldehyde dehydrogenase 1 family member A3 (ALDH1A3) can promote PDAC transfer by affecting glucose metabolic pathways [46]. Furthermore, the interaction between HK2 and ALDH1A3 was confirmed, that is, both proteins were expressed in cytoplasm and their spatial positions were highly consistent. In addition, clinical retrospective studies showed that patients with high expression of ALDH1A3 and HK2 usually had poor prognosis, and their overall survival period and five-year survival rate were significantly lower than those with low expression [47].

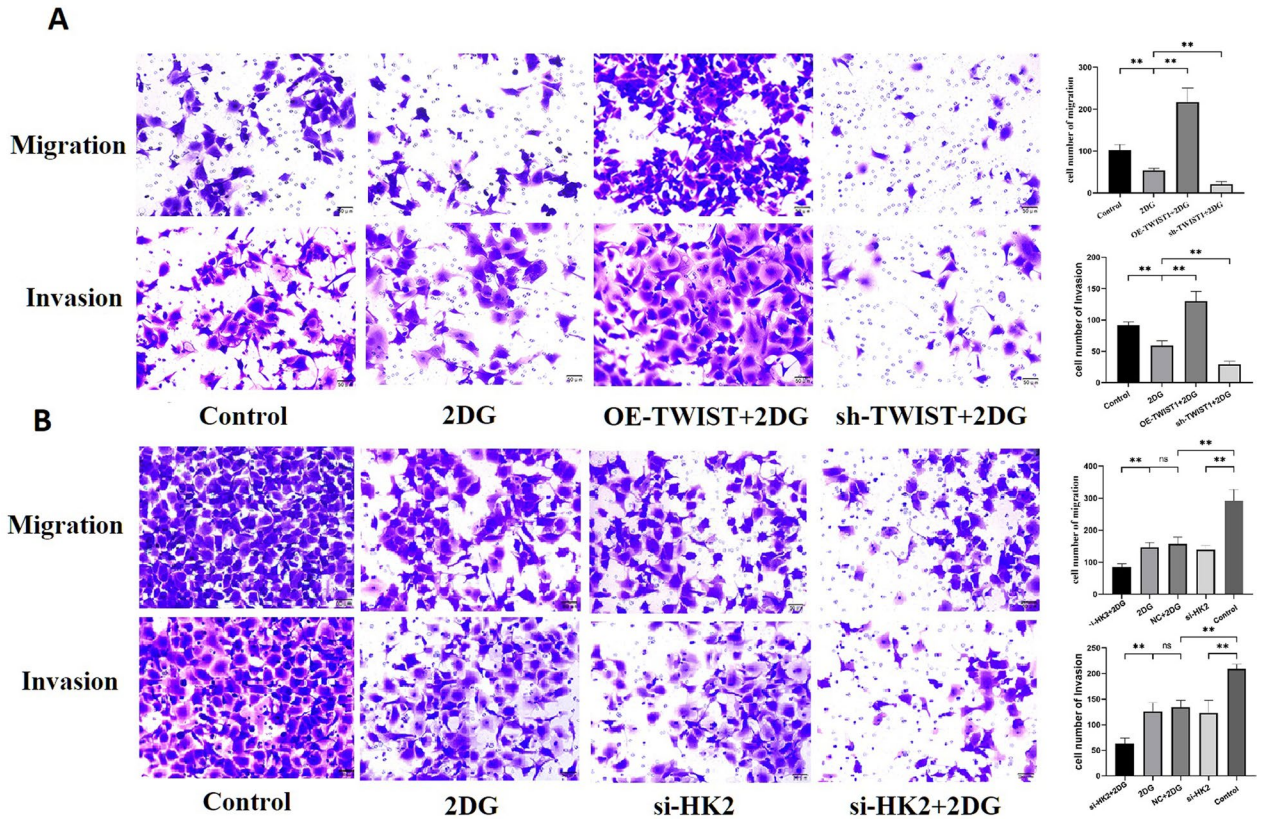

Fig. 7 Effects of glycolysis on cancer cell migration and invasion **A**. Transwell assay detected the migration and the invasion of cancer cell that treated by 2DG and transfected by shTWIST1. **B**. Transwell assay detected the migration and the invasion of cancer cell that treated by 2DG and transfected by si-HK2

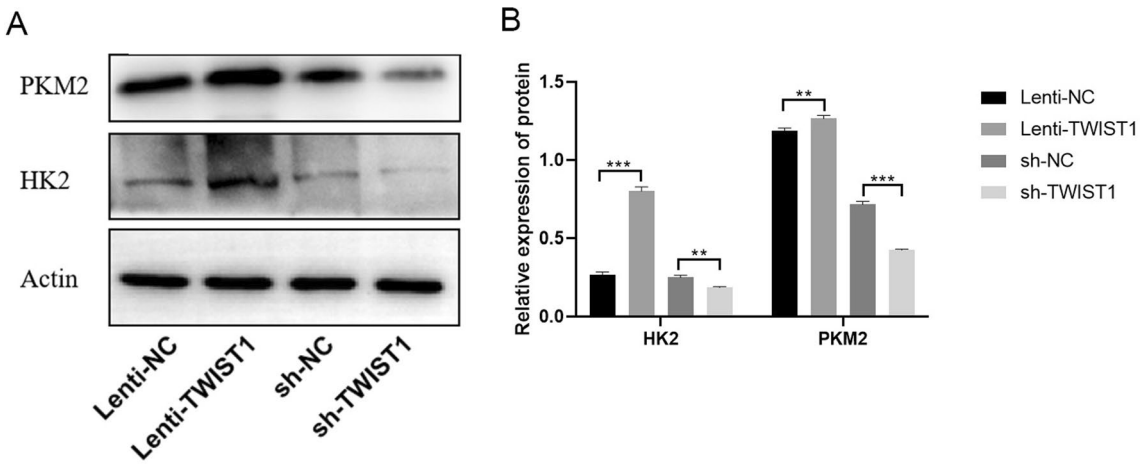

Fig. 8 The interaction between TWIST1, HK2 and PKM2. over-expression of TWIST1 induced increasing expression of HK2 and PKM2. Inhibition of TWIST1 caused decrease of HK2 and PKM2 expression. **A**, **B**. Western blot and analysis of PKM2 and HK2

In recent years, TWIST1 and HK2 were both proved to be essential for the tumor progression, however, the underlying mechanism, especially in PDAC. Our previous study suggested that TWIST1 could directly increase the expression of HK2, so we focused on the relationship between these two proteins and their tumorigenesis role

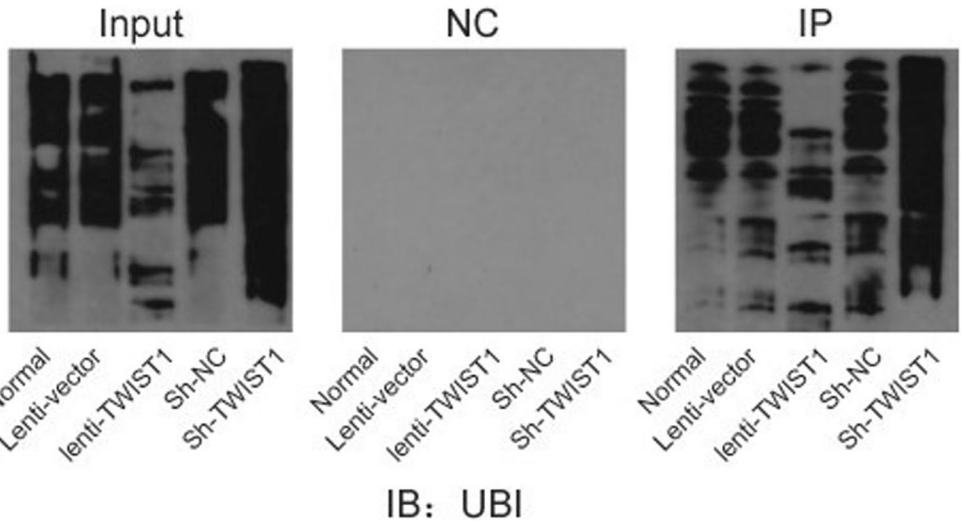

Fig. 9 TWIST1 inhibits the ubiquitination degradation of HK2

as well as underlying mechanism in PDAC. In this study, we proved that mRNA and protein levels of TWIST1 and HK2 elevated dramatically in PDACs, which indicated that TWIST1 and HK2 were major regulators of aerobic glycolysis in PDACs. And there is a positive correlation between TWIST1 and HK2. Besides, we found that TWIST1 could directly bind with HK2 and stabilize its function via inhibiting the ubiquitination degradation process of HK2.

The glycolytic pathway often leads to cuproptosis, which is also key to promoting cancer cell metastasis. Here we observed whether TWIST1 promotes cell invasion and migration through the HK2-induced cuproptosis pathway. Sh-TWIST1 inhibited expression of cuproptosis related gene FDX1, while over-expression promoted FDX1 expression. By knocking down FDX1, invasion and migration were inhibited. In examining the effects of cuproptosis and glycolysis on cancer cell migration and invasion, we found that Elesclomol-Cu and 2DG alone or together treated significantly inhibited migration and invasion.

Limitations

The limitations of this study are as follows: The study utilized a relatively small sample size (39 patients), which may limit the generalizability of the findings. Larger sample size studies are needed to validate these results. Most of the experiments were conducted in vitro using cell lines. While these provide valuable insights into the molecular mechanisms, in vivo studies are necessary to confirm these findings in a more complex biological environment. The study mainly focused on the interaction between TWIST1 and HK2, potentially overlooking other key players in glycolysis and pancreatic cancer progression. Further research could explore other molecular pathways involved in this process. Although the study demonstrated a correlation between TWIST1/HK2 expression and pancreatic cancer progression, it did not establish a direct cause-and-effect relationship in clinical settings. Prospective clinical trials would be necessary to establish this link.

In summary, based on the above results, we proved that TWIST1 could enhance glycolysis of pancreatic cancer cells by promoting HK2 expression, thereby promoting the invasion and metastasis of pancreatic cancer. And confirmed TWIST1 regulates HK2 ubiquitination degradation to promote cuproptosis caused by cell glycolysis and promote pancreatic cancer invasion and metastasis. Furthermore, the results of this study may conduce to accelerate the development of new treatment schemes for pancreatic cancer.

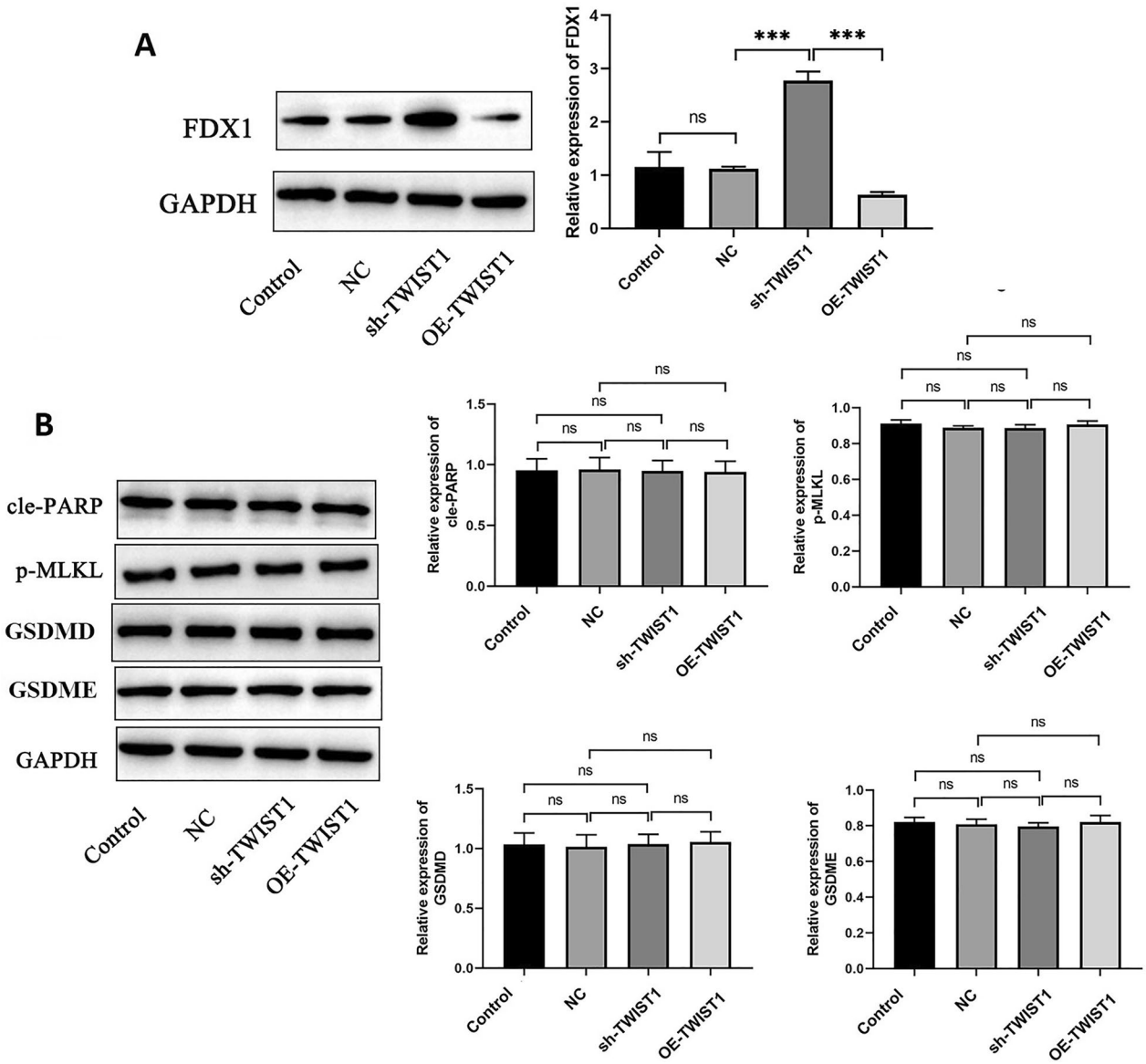

Fig. 10 TWIST1 regulates HK2 to promote cuproptosis triggered by cellular glycolysis **A**. The expression of cuproptosis related protein FDX1 was detected by WB. **B**. Western Blotting to detect the expression of cle-PARP, p-MLKL, GSDMD and GSDME.)

Supplementary Information

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Supplementary material 1

Supplementary material 2

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Author contributions

Conceptualization, resources and project administration, X.X.W., Z.H.Z. and M.Z.M.; methodology and software, S.S., X.W.X., C.Q. and R.X.G; validation and formal analysis, M.Z.M., S.S. and X.W.X; investigation, C.Q. and R.X.G; data curation and visualization, Z.H.Z. and M.Z.M.; writing—original draft preparation, X.W.X., C.Q. and R.X.G.; writing—review and editing, X.X.W. and Z.H.Z. supervision, X.X.W.; funding acquisition, Z.H.Z. All authors have read and agreed to the published version of the manuscript.

Availability of data and materials
Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
All patients provided written informed consent before enrollment, and this study was approved by the Research Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University.

Competing interests
The authors declare no competing interests.

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