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PDZK1 in Cholangiocarcinoma: Suppression of pAkt-Induced Glycolysis and Tumor Progression under Hypoxia

Yuangan Chen, Zhi Li

The Second Affiliated Hospital of Anhui Medical University, Hefei City, Anhui Province, China

Abstract: *PDZ-containing 1 (PDZK1) functions as a tumor suppressor in various cancers, but its role in cholangiocarcinoma (CCA) remains unclear. This study investigated the involvement of PDZK1 in CCA progression, particularly under hypoxic conditions that mimic late-stage tumors. Using lentiviral-mediated overexpression in CCLP and RBE cells, we assessed cell proliferation, apoptosis, migration, and glycolysis through CCK8, flow cytometry, wound healing assays, and measurements of glucose consumption, ATP, and lactate production. Hypoxia enhanced glycolytic activity and malignant behaviors while reducing PDZK1 expression. Overexpression of PDZK1 reversed these hypoxia-induced effects, suppressing proliferation, migration, and glycolysis, and promoting apoptosis. Mechanistically, PDZK1 overexpression downregulated hexokinase 2 (HK2) expression and activity, accompanied by reduced Akt phosphorylation. Activation of pAkt by perifosine partially abrogated the inhibitory effects of PDZK1 on glycolysis. These findings demonstrate that PDZK1 attenuates CCA progression under hypoxia by suppressing pAkt-mediated glycolysis, highlighting its potential as a therapeutic target for advanced CCA.*

Keywords: *Cholangiocarcinoma, PDZK1, glycolysis, hypoxia, HK2, pAkt*

1. Introduction

Cholangiocarcinoma (CCA) is a type of cancers with aggressive malignancy and poor prognosis [1]. Due to lack of obvious and specific symptoms, signs and diagnostic markers [2-4], more than 50% patients diagnosed as CCA are at late stage. Therefore, those patients are not suitable for surgical resection, but can only receive gemcitabine plus cisplatin as therapeutic options [5, 6]. Therefore, identifying a therapeutic target for late stage CCA is definitely required.

Consistent increased glycolysis has been found in cancer cells, which generates large amount of energy and lactate, supports tumor growth and metastasis and induces immune escape [7, 8]. During glycolysis, there exist three rate-limiting enzymes which are hexokinases (HKs), phosphofructokinase (PFK) and pyruvate kinase (PK) [9, 10]. Among these enzymes, HKs are the first rate-limiting enzyme switching the process of glycolysis. Till now, five HK isoforms (HK1-4 and HKDC1) have been confirmed in human [11,12]. Targeting glycolysis has been identified as a hopeful method for tumor treatment [13]. For example, Sun et al. have reported that reversal of the glycolysis attenuates metastatic breast cancer cell growth [14]. These evidence highlights the importance of mining mechanism regulating glycolysis.

PDZ-containing 1 (PDZK1) is a new binding protein related to PTEN [15]. Previous studies have revealed that FANCI participates in the progression of tumors, including renal cell carcinoma and breast cancer [16, 17]. However, the role of PDZK1 in CCA, especially in CCA at late stage, remains unknown. Therefore, exploring the role of PDZK1 in CCA at late stage is beneficial for identifying a potential target for CCA treatment.

This study hired hypoxia to partly mimic the growing condition of CCA cells, and compared the biological feature of CCA cells under hypoxia and normoxia. Then, we explored genes with changed level under hypoxia and analyzed its role in progression and glycolysis of CCA cells. Finally, we explored the mechansim of PDZK1 in CCA cells in regulating glycolysis.

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2. Methods

2.1 Cell culture and drug intervention

CCA cell lines (CCLP and RBE) were gifts obtained from Renji Hospital of Shanghai Jiaotong University, which were cultured in RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) and penicillin/streptomycin at 37 ℃ with 5% CO2. For hypoxia, cells were cultured with 1% O2. Perifosine (Selleck, USA) was added into the medium to enhance the phosphorylation of Akt.. In detail, 45 μmol/L perifosine were used and cells were incubated for 72 hours to active Akt[18]. 2-deoxy-D-glucose (MCE, New Jersey, USA) were used and cells were incubated for at least 18 hours to inhibit glycolysis[19].

2.2 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from CCLP and RBE cells through TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and then reversed to cDNA by PrimeScript RT Reagent Kit (Progema, Madison, Wisconsin, USA). The qualification of PDZK1, HK2, PFK1, PKM2, LDHA and LDHB was performed using SYBR Green (Takara). All these experiments were conducted according to the manufacture's protocol provided. The cycle time (Ct) values were normalized to β-actin and the qualification of genes was analyzed by the 2-ΔΔCt method. The primers used here were listed in Table 1.

Table 1: Primer sequences used for quantitative real-time PCR.

Gene Symbol	Forward Primer (5'→3')	Reverse Primer (5'→3')
PDZK1	TTCCTGCGAATTGAGAAGGAC	TCCACCCGTGTTTT CACTGC
HK2	ATGATCGCCTGCTTATTCACG	CGCCTAGAAATCTCCAGAAGGG
PFK1	AGCGTTTCGATGATGCTTCAG	GGAGTCGTCCTTCTCGTTCC
PKM2	GGGCCATAATCGTCCTCACC	TTGCACAGCACAGGGAAGAT
LDHA	ATTAAGCTGTCATGGGTGGGTC	CAGAGAGACACCAGCAACATTCA
LDHB	TGCGTCCGTTGCAGATGAT	TTTCGGAGTCTGGAGGAACAA
β-actin	AGGGAAATCGTGCGTGAC	CGCTCATTGCCGATAGTG

2.3 Western Blotting

Western blotting was carried out as previous [20]. In brief, mitochondrial protein or total protein was obtained from CCLP and RBE cells and quantified with BCA Protein Assay Kit (Sangon Biotech, Shanghai, China). Proteins were resolved and then transferred to polyvinylidene difluoride (PVDF) membranes. Primary antibodies were loaded onto the membranes and blocked for 2 hours. Then, primary antibodies were incubated at 4 °C overnight. After that, secondary antibodies were added and incubated for 2h at 37°C. The electrophoresis bands were visualized by the enhanced chemiluminescence (ECL) detection kit (ThermoFisher, Waltham, MA, USA). The primary antibodies for proteins used in this study were anti-PDZK1 (ab92491, Cambridge, UK), anti-Akt (ab108202, Cambridge, UK), anti-pAkt (ab38449, Cambridge, UK) and anti-HK2 (ab209847, Cambridge, UK).

2.4 Construction of stable PDZK1 over-expression CCA cells

PDZK1 was over-expressed in CCLP and RBE cells (OE-PDZK1). This procedure was conducted as previously described [21]. In brief, lentiviral vector was infected into CCLP and RBE cells and incubated for 48 hours. Then, puromycin (5 μg/mL) was added to filter cells with stable PDZK1 over-expression.

2.5 Cell Counting Kit-8 (CCK-8) Assay

CCK-8 assay was carried out using cell counting kit (Beyotime, Shanghai, China) based on the manufacture's protocol. In detail, CCLP and RBE cells were seeded into 96-well plates (2×103 cells/well in 100 μl culture medium). 10 μl CCK-8 reagent was added into each well after for 5 consecutive days after 24 hours. Absorbance of cells at 450 nm wavelength was measured from day 1 to 5 after being incubated at 37 °C in dark for 2.5 hours.

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2.6 Wound Healing Assay

CCLP and RBE cells were seeded into 12-well plate and cultured to 90% confluence. Then, 200 μl plastic pipette tip were used to scratch. After that, cells were cultured with 5% FBS and the status of the scratches were recorded at 24h.

2.7 Cell apoptosis assays

Annexin-V FITC/PI double staining method was used to measure the apoptosis of fixed CCLP and RBE cells. In detail, 300 μl fix buffer was added to fix cells. 5 μl staining reagent was then added and incubated in the dark for 5 minutes at room temperature. Finally, flow cytometry (FACS) machine was used to detected the apoptotic situation of cells.

2.8 Glucose colorimetric assay

Glucose in cell supernatant was measured using glucose colorimetric assay kit (Elabscience, Wuhan, China). Firstly, 3 μl sample was added into a 96-well plate. Then 300 μl enzyme working solution was added and the plate was incubated at 37 ℃ for 15 min. Finally, absorbance at 505 nm wavelength was measured.

2.9 ATP colorimetric assay

ATP concentration was measured as the manufacture's protocol provided in the ATP colorimetric assay kit (Elabscience, Wuhan, China). Cells were scraped and cell suspension was collected. Then, the supernatant was discarded after centrifuging the cell suspension at 1000 g for 10 min at 4 ℃. After that, 30 μl sample, 330 μl working solution and 330 μl working solution was subsequently added into a 1.5 ml tube. The mixture was subsequently centrifuged at 10000 g for 5 min, and 300 μl supernatant was taken for determination. Further, 500 μl chromogenic agent was added into the tube and incubated at room temperature for 2 minutes. Finally, 500 μl Stop Solution was added and absorbance at 636 nm wavelength was measured using quartz cuvette after incubating at room temperature for 5 minutes.

2.10 L-lactic acid colorimetric assay kit

The concentration of l-lactic acidwas measured as the manufacture's protocol provided in the lactic acid colorimetric assay kit (Elabscience, Wuhan, China). 5 μl sample, 100 μl enzyme working solution and 20 μl chromogenic agent were subsequently added into a 96-well plate and incubated the mixture at 37 ℃ for 10 min. The absorbance at 530 nm wavelength was measured after adding 180 μl stopping buffer.

2.11 HK activity assay

HK activity was calculated using the amount of NADPH production determined by Hexokinase activity assay kit (NJJCBIO, Nanjing). In short, mix whole-cell extract and assay mixture efficiently. The absorbance of the mixture at 530 nm wavelength was measured to determine the baseline. Then glucose-6-phosphate dehydrogenase was added and the absorbance was measured every 2.5 min for three times.

2.12 Download and process data from GEO database

Transcript data of CCA was downloaded from GSE26566 (Platform: GPL6104) and GSE107943 (Platform: GPL18573). GSE26566 includes 106 tumor tissues and 63 non-tumor tissues, and GSE107943 includes 30 tumor tissues and 27 non-tumor tissues. Limma package was used for data analysis.

2.13 Statistical Analysis

Prism 7 (GraphPad Software, La Jolla, CA, USA) was hired for data analysis. All experiments were repeated for three times, and all data were presented as mean±S.D (Standard deviation). Unpaired two-tailed Student's t-test was performed to detect two group differences and one-way analysis of variance (ANOVA) with Bonferroni's post-hoc test for multiple comparisons. Mann-Whitney U test

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was employed to analyze data obtained from GEo database. P value < 0.05 were regarded as statistically significant.

3. Results

3.1 Glycolysis of CCA cells increased under hypoxia

Hypoxia is a common phenomenon in tumor. To verify the effect of hypoxia on glycolysis, we cultured CCLP and RBE cells under hypoxia and normoxia and compared their glycolysis status. Firstly, we measured the expression of key enzymes of glycolysis. As shown in Figure 1A, the mRNA levels of HK2, PFK1, PKM2 and LDHA dramatically increased under hypoxia, while the expression of LDHB decreased under hypoxia. Further, we detected the glucose consumption, ATP production and extracellular lactate in these cells. The results indicated that cells consume more glucose and generate more ATP and lactate under hypoxia (Figure 1B-D). Taken together, hypoxia is an enhancer promoting glycolysis in CCA cells.

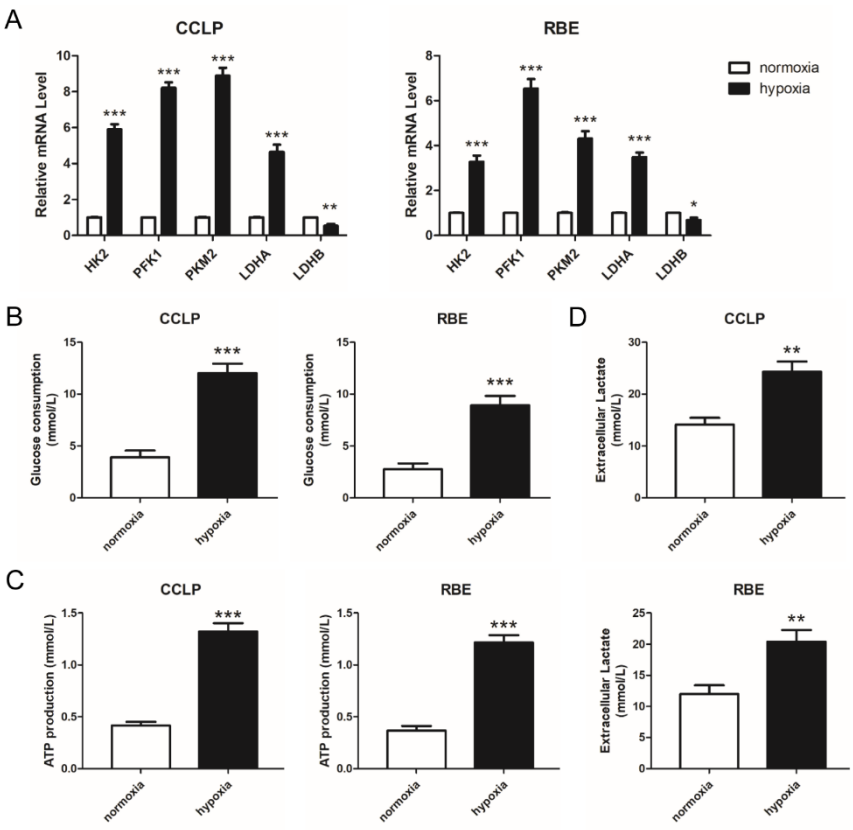

*Figure 1: Glycolysis of CCA cells increased under hypoxia. (A) the mRNA levels of HK2, PFK1, PKM2, LDHA and LDHB in CCLP and RBE cells under hypoxia and normoxia. (B) The glucose consumption, (C) ATP production, and (D) lactate production of CCLP and RBE cells under hypoxia and normoxia. *P<0.05, **P<0.01, ***P<0.001.*

3.2 Glycolysis enhanced by hypoxia was a driver promoting CCA progression

To detect the effect of glycolysis and hypoxia on CCA malignancy. We here compared the bio-function of cells cultured under hypoxia with or without 2-DG which could inhibit the activity of HK2, and normoxia. We firstly chose a proper concentration of 2-DG for experiments, and the production of extracellular lactate and cell viability were selected as criteria. The effect of 2-DG on inhibiting glycolysis showed dose dependent manner to a certain degree (Figure 2A). However, considering cell viability (data not shown), the final concentration of 2-DG used in this study was 5 mM. Then, we compared the proliferation and apoptosis of these cells. Results of CCK8 assay showed that the OD450/Fold Change value of cells cultured under normoxia is about 1.5 to 2 times that of cells cultured under hypoxia at day 5, and the OD450/Fold Change value keeps stably in cells cultured with

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2-DG (Figure 2B). Also, more apoptotic cells were detected under normoxia and hypoxia with 2-DG compared to hypoxia without 2-DG(Figure 2C). Finally, we compared the migrative abilities of cells in these three groups. As shown in Figure 2D, much more cell migrated under hypoxia without 2-DG added compared to the other two groups. Given the above, hypoxia induced enhancement of glycolysis is indispensable for CCA progression.

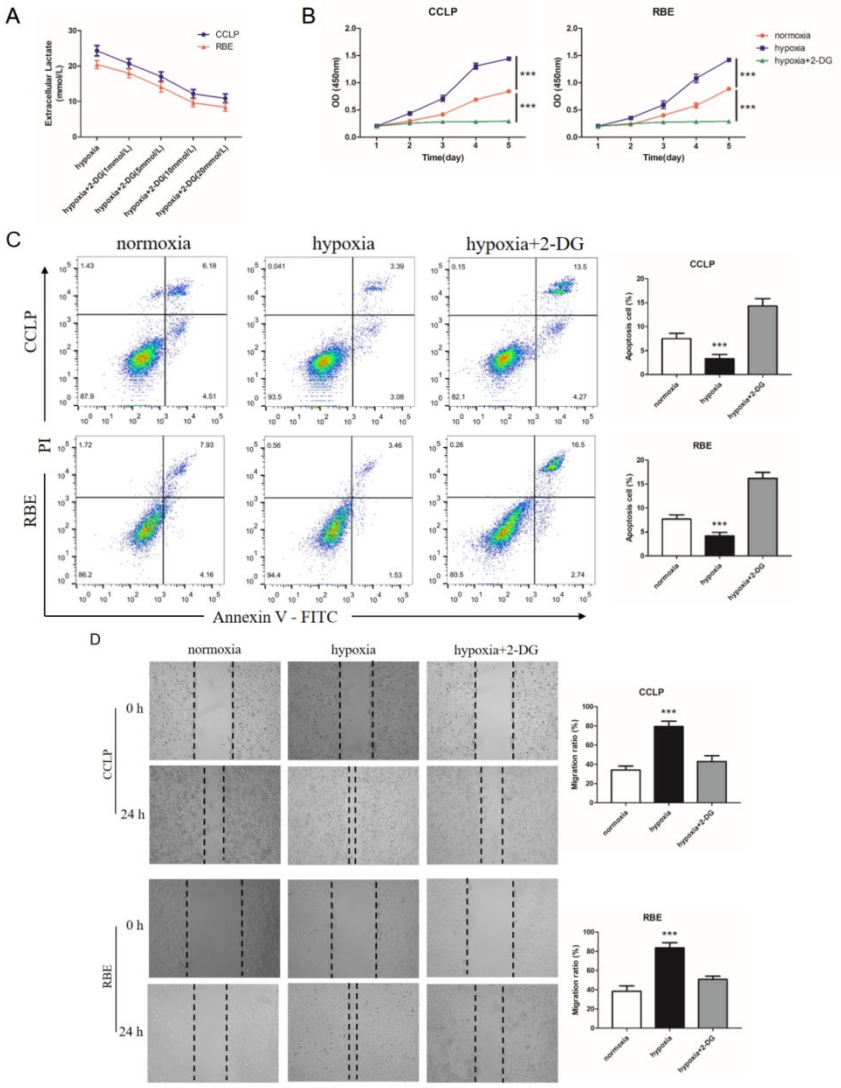

*Figure 2: Glycolysis enhanced by hypoxia was a driver promoting CCA progression. (A) The effect of 2-DG on glycolysis in CCLP and RBE cells. (B) The OD450/Fold Change value of CCLP and RBE cells under normoxia and hypoxia with or without 2-DG determined by CCK8 assay. (C) The apoptotic status of CCLP and RBE cells under normoxia and hypoxia with or without 2-DG determined by FACS. (D) The migrative abilities of CCLP and RBE cells under normoxia and hypoxia with or without 2-DG. ***P<0.001.*

3.3 PDZK1 could reverse the effect of hypoxia on CCA progression

Because PDZK1 is a tumor suppressor confirmed in several tumors previously, we here selected PDZK1 as the target we studied.To identify the effect of hypoxia on PDZK1 expression, we firstly measured the mRNA and protein levels of PDZK1 in CCLP and RBE under hypoxia and normoxia. As shown in Figure 3A/B, levels of PDZK1 decreased in both CCLP and RBE under hypoxia. Then, we analyzed the proliferation and apoptosis of CCLP and RBE cells after PDZK1 over-expression under hypoxia. Results of CCK8 assay showed that the OD450/Fold Change value of NC-PDZK1 CCLP and RBE cells are about 1.5 to 2 times that of OE-PDZK1 CCLP and RBE cells at day 5 (Figure 3C). Also, significantly less apoptotic cells were found in NC-PDZK1 CCLP and RBE cells compared to OE-PDZK1 CCLP and RBE cells (Figure 3D). Finally, migrative abilities were performed on cells in the two groups. As shown in Figure 3E, migrated CCLP and RBE cells were markedly reduced in

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OE-PDZK1 group compared to NC-PDZK1 group. Taken together, hypoxia caused decreased PDZK1 expression, however over-expression of PDZK1 could reverse the effect of hypoxia on CCA progression.

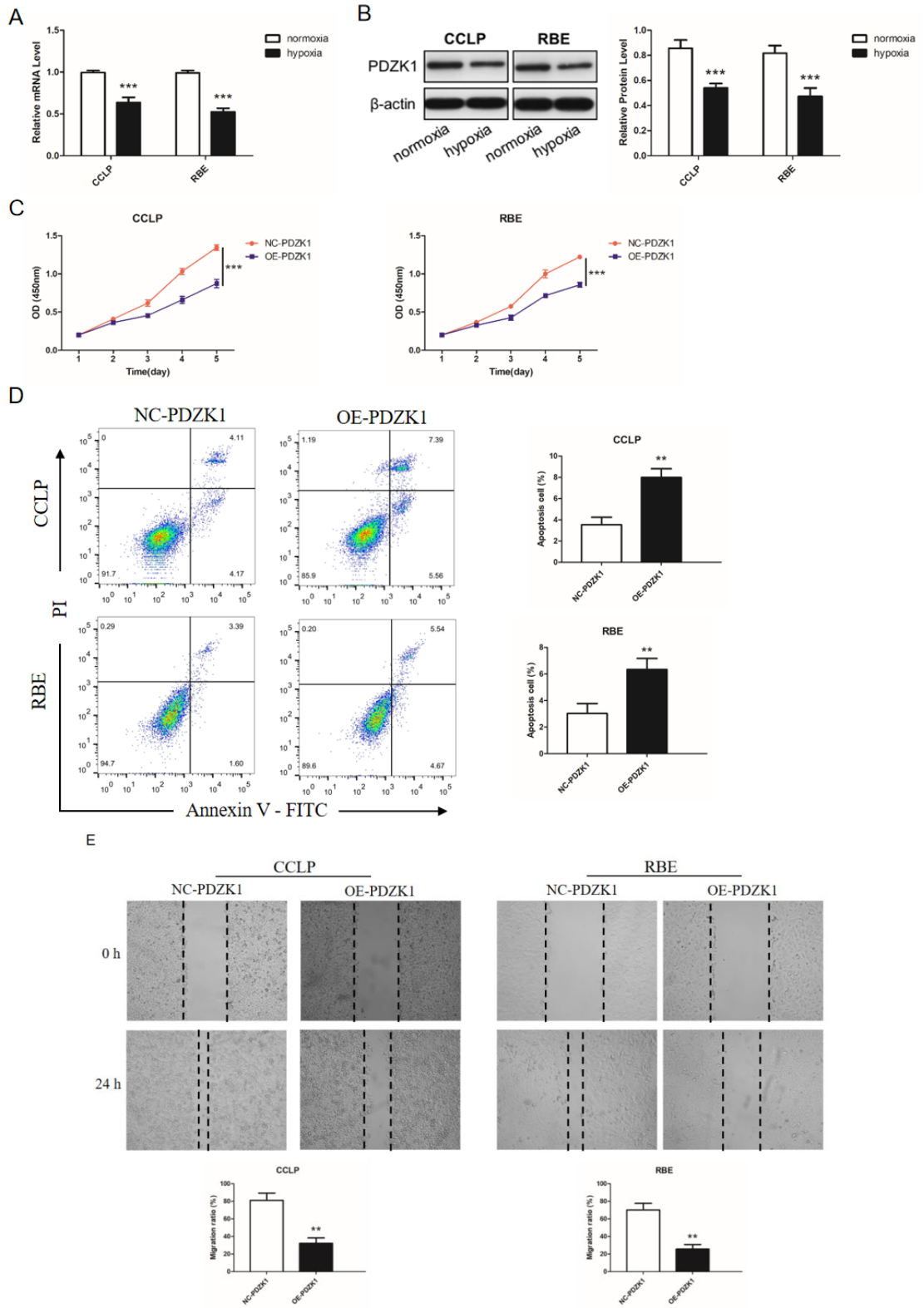

*Figure 3: PDZK1 could reverse the effect of hypoxia on CCA progression. (A) The mRNA and (B) protein level of PDZK1 in CCLP and RBE under hypoxia and normoxia. (C) The OD450/Fold Change value of CCLP and RBE cells with or without PDZK1 over-expression. (D) The apoptotic status of CCLP and RBE cells with or without PDZK1 over-expression. (E) The migrative abilities of CCLP and RBE cells with or without PDZK1 over-expression. *P<0.05, **P<0.01, ***P<0.001.*

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3.4 PDZK1 attenuated glycolysis in CCA

We compared the intensity of glycolysis in cells with or without PDZK1 over-expression under hypoxia. The results indicated that the glucose consumption, ATP production and lactate production of OE-PDZK1-CCLP cells were significantly decreased compared with NC-PDZK1-CCLP cells, respectively. Similarly, the glucose consumption, ATP production and lactate production of OE-PDZK1-RBE cells were noticeably decreased compared with NC-PDZK1-RBE cells, respectively (Figure 4A-C). In a word, PDZK1 inhibits glycolysis in CCA cells.

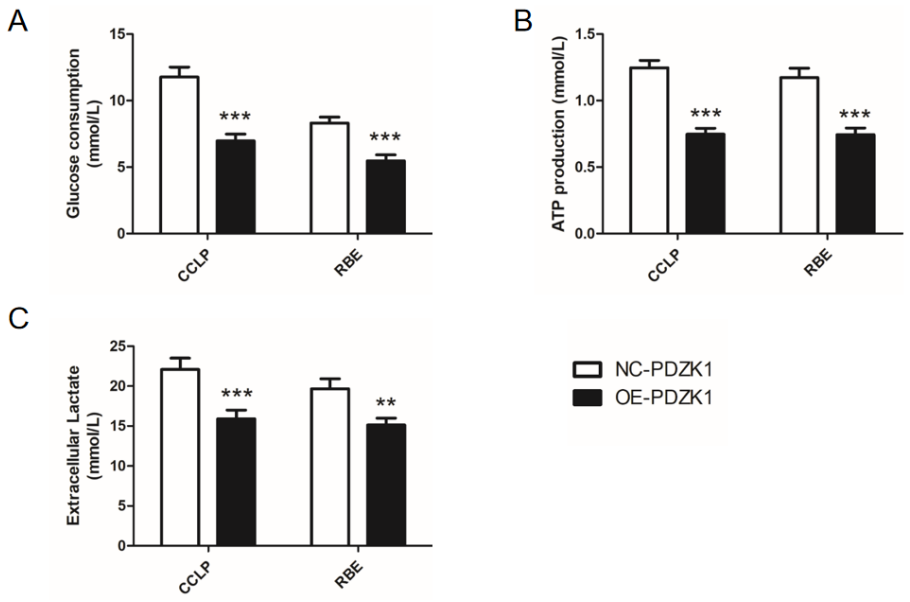

*Figure 4: PDZK1 attenuated glycolysis in CCA. The glucose consumption, (B) ATP production, and (C) lactate production of CCLP and RBE cells with or without PDZK1 over-expression. *P<0.05, **P<0.01, ***P<0.001.*

3.5 PDZK1 induced the release of HK2 from the mitochondria in CCA

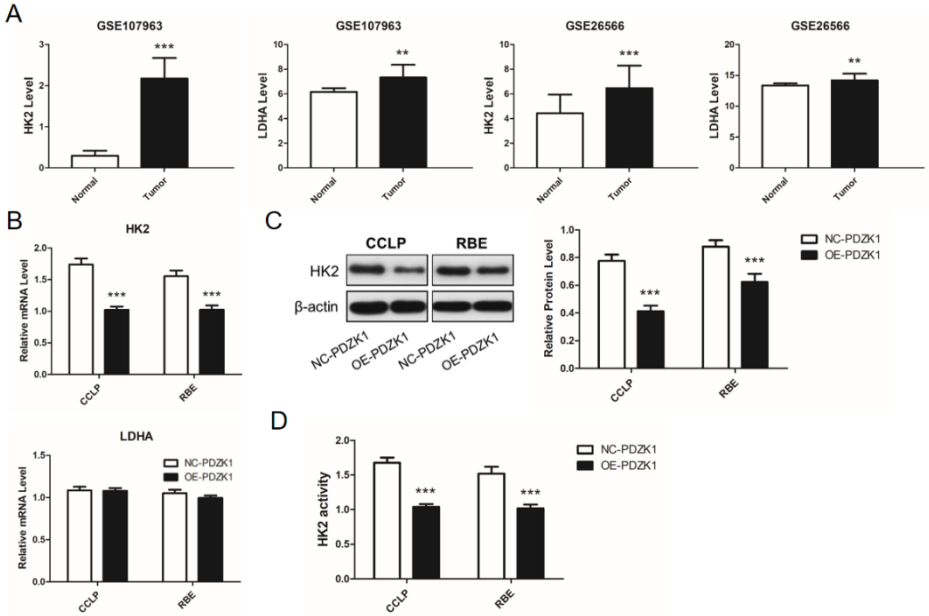

*Figure 5: PDZK1 induced the release of HK2 from the mitochondria in CCA. (A) Expression of HK2 and LDHA in GSE107943 and GSE26566 databases. (B) mRNA and (C) protein expression of HK2 in CCLP and RBE cells with or without PDZK1 over-expression. (D) HK2 activity in CCLP and RBE cells with or without PDZK1 over-expression. *P<0.05, **P<0.01, ***P<0.001.*

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To find the targets of PDZK1 in regulating glycolysis, we firstly analyzed the expression of key enzymes of glycolysis in CCA, including HK2, PFK1, PKM2, LDHA, LDHB, through GEO database. The results obtained from GSE107943 and GSE26566 demonstrated that only HK2 and LDHA levels increased in tumor tissues compared to non-tumor tissues (Figure 5A). However, the expression of other molecules was similar in tumor and non-tumor tissues (Data not shown). Then, we analyzed the mRNA level of HK2 and LDHA in OE- and NC-PDZK1 cells, and the results showed that only HK2 expression changed dramatically (Figure 5B). We also compared the protein expression of HK2 in mitochondrial proteins extracted from CCLP and RBE cells with or without PDZK1 over-expression, and decreased HK2 level was detected in OE-PDZK1 cells(Figure 5C). Further, we compared HK2 activity in NC-PDZK1 and OE-PDZK1 groups, and we found that HK2 activity attenuates by approximately 40% in OE-PDZK1 group (Figure 5D). Overall, PDZK1 weakened glycolysis in CCA cells through down-regulating HK2 expression and activity.

3.6 PDZK1 promotes the disassociation of HK2 from mitochondria through attenuating phosphorylation of Akt

We here analyzed the phosphorylation status of Akt in NC-PDZK1 and OE-PDZK1 cells, and found that Akt phosphorylation decreased in OE-PDZK1 cells (Figure 6A). To explore whether pAkt is the medium of PDZK1 regulating HK2, we employed perifosine, an activator of Akt. We found that the expressive level of HK2 restored after using perifosine in OE-PDZK1 cells (Figure 6B). Further, perifosine reverse the effect of PDZK1 on inhibiting glycolysis in CCA cells. In detail, the glucose consumption, ATP production and lactate production of CCLP cells with perifosin were significantly higher than those of CCLP cells without Perifosin, respectively. Also, the glucose consumption, ATP production and lactate production of RBE cells with perifosin were prominently increased compared to those of RBE cells without Perifosin, respectively (Figure 6C-E). Overall, PDZK1 negatively regulates HK2 through inhibiting phosphorylation of Akt.

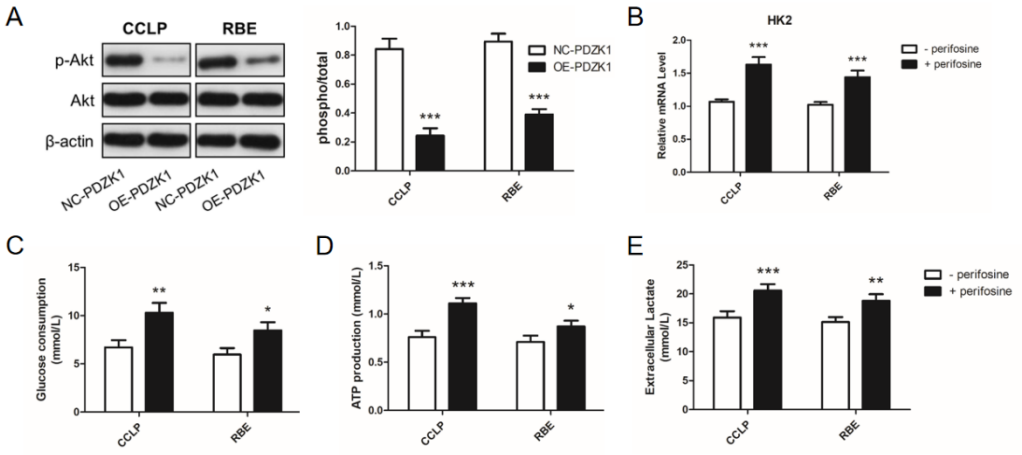

*Figure 6: PDZK1 promotes the disassociation from mitochondria through attenuating phosphorylation of Akt. (A) The level of pAkt and Akt in CCLP and RBE cells with or without PDZK1 over-expression determined by western blotting. (B) The level of HK2 mRNA in OE-PDZK1 CCLP and RBE cells with or without perifosine. (C) The glucose consumption, (D) ATP production, and (E) lactate productionin in OE-PDZK1 CCLP and RBE cells with or without perifosine. *P<0.05, **P<0.01, ***P<0.001.*

4. Discussion

Patients with CCA at late stage can only receive gemcitabine plus cisplatin as first-line treatment. So far, there exist no standard second-line therapeutic options and supplement drugs for first-line treatment. Mining a novel target for CCA could be a supplement for the treatment of CCA at late stage.

One of the most significant features of tumor in late stage is lack of oxygen. Therefore, to mimic CCA in late stage, we here hired hypoxia (1% O2) as culture condition for CCA cells. This method had been used widely in previous researches [22]. After culturing CCA cells under hypoxia for 48 hours, we compared the biological function of CCA cells, and found that CCA cells exhibit aggressive malignancy. Moreover, the effect of hypoxia was induced by enhanced glycolysis under hypoxia. These finding are similar to previously reported results [23, 24]. Above evidence demonstrated the importance

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of glycolysis in CCA. Therefore, identifying molecules inhibiting glycolysis is vital.

PDZK1 is a tumor suppressor confirmed in several type of cancers. In view of its function in tumor, we selected it as target studied in this study. We firstly compared the expression of PDZK1 in CCA cells under hypoxia and normoxia, and found the level of PDZK1 decreased in cells under hypoxia. Then, we found that PDZK1 attenuates CCA progression. These results are consistent with the results reported previously in which PDZK1 was identified as a tumor suppressor [16, 17]. Further, we compared the glycolytic status of CCA cells with or without PDZK1 over-expression, and found that PDZK1 suppresses glycolysis of CCA cells. Combing these results together, we concluded that PDZK1 inhibits CCA progression through suppressing glycolysis under hypoxia.

To identify the targets of PDZK1 inhibiting glycolysis, we compared the level of rate-limiting enzyme of glycolysis in tumor and non-tumor tissues, and in CCA cells with or without PDZK1 over-expression. We found that level of HK2 is lower in normal tissues and cells with PDZK1 over-expression. Also, we compared the activity of HK2 in CCA cells with or without PDZK1 over-expression, and found a decreased activity of HK2 in CCA cells after up-regulating PDZK1. Previous studies have confirmed that HK2 expression and activity can be regulated by p53, Akt and myc [10]. Among them, pAkt acts as an oncogene in numerous cancers. Besides, PDZK1 is a serine/threonine phosphatase, which poses phosphatase activity [15]. Therefore, we hypothesized that PDZK1 may inhibits HK2 activity and expression through attenuating phosphorylation of Akt. Here, we firstly detected the phosphorylation status of Akt in CCA cells with or without PDZK1 over-expression and found that pAkt decreased in CCA cells with PDZK1 over-expression. Further, we employed an Akt activator, perifosine, in CCA cells and found that the effect of PDZK1 over-expression on CCA cells can be reversed by perifosine. Taken together, PDZK1 can inhibits HK2 activity and expression through attenuating phosphorylation of Akt.

There exist several limitations in this study. On one hand, we failed to confirmed the results in mice. On other hand, we had not detected the level of PDZK1, pAkt and HK2 in fresh CCA tissue.

In conclusion, PDZK1 suppresses CCA progression through attenuating pAkt induced glycolysis.

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