Original Article

The mTOR Pathway Regulates PKM2 to Affect Glycolysis in Esophageal Squamous Cell Carcinoma

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Abstract

Objectives: Esophageal squamous cell carcinoma is a highly prevalent cancer withpoor survival rate and prognosis. Increasing evidence suggests an important role for metabolic regulation in treating esophageal squamous cell carcinoma, but the underlying mechanism remains unclear. The pyruvate kinase M2 isoform is a key enzyme in the energy production process, and the upregulation of pyruvate kinase M2 isoform also plays a crucial role in gene transcription and tumorigenesis. The mammalian target of rapamycin pathway regulates an array of cellular functions, including protein synthesis, metabolism, and cell proliferation. The pyruvate kinase M2 isoform and mammalian target of rapamycin pathways both affect metabolism in cancers, and evidence also suggests that the mammalian target of rapamycin downstream transcription factor hypoxia-inducible factor-1α regulates pyruvate kinase M2 isoform. We therefore investigated the regulatory mechanism among pyruvate kinase M2 isoform, mammalian target of rapamycin, and aerobic glycolysis in esophageal squamous cell carcinoma, hoping to prove that mammalian target of rapamycin pathway regulates pyruvate kinase M2 isoform to affect glycolysis in esophageal squamous cell carcinoma. **Methods:** Immunohistochemical staining was used to compare pyruvate kinase M2 isoform and phospho-mammalian target of rapamycin expression in 30 human pathological esophageal squamous cell carcinoma sections and 30 nontumoral esophageal tissues. Short hairpin RNA was used to inhibit pyruvate kinase M2 isoform and activate mammalian target of rapamycin, after which we monitored changes in glucose consumption and lactate production. Finally, we determined the expression of pyruvate kinase M2 isoform and the mammalian target of rapamycin downstream transcription factor hypoxia-inducible factor-1α, as well as glucose consumption and lactate production, following the modification of mammalian target of rapamycin expression. **Results:** Immunohistochemical staining showed that both phospho-mammalian target of rapamycin and pyruvate kinase M2 isoform expression were higher in esophageal squamous cell carcinoma than in nontumor tissues. Glucose consumption and lactate production measurements demonstrated that altering mammalian target of rapamycin and pyruvate kinase M2 isoform levels caused corresponding changes in glycolysis in esophageal squamous cell carcinoma cells. When mammalian target of rapamycin was activated or inhibited, expression of pyruvate kinase M2 isoform and hypoxia-inducible factor-1α as well as glycolysis were altered, indicating that mammalian target of rapamycin regulates pyruvate kinase M2 isoform via the downstream transcription factor hypoxia-inducible factor-1α, thereby affecting glycolysis in esophageal squamous cell carcinoma. **Conclusion:** Mammalian target of rapamycin pathway promotes aerobic glycolysis in esophageal squamous cell carcinoma by upregulating pyruvate kinase M2 isoform. Both proteins can serve as molecular targets for novel therapeutic strategies.

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Introduction

Esophageal cancer (EC) is ranked as the eighth most prevalent cancer and is the sixth leading cause of cancer-related deaths worldwide.[1] The EC is among the leading causes of cancer death in China, with an estimated 4 292 000 new cancer cases and 2 814 000 cancer deaths occurred in China in 2015.[2] Esophageal squamous cell carcinoma (ESCC) is one of the primary pathological subtypes of EC. The ESCC is predominant in East Asia and accounts for 95% of all Chinese patients with EC,[3,4] showing poor survival,[5] and considerable efforts have been made to detect the mechanisms that underlie the genesis, development, invasion, and metastasis of ESCC.

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is activated by several oncogenic signaling pathways and is accordingly hyperactive in a majority of cancers,[6] serving as a core kinase.[7] The mTOR belongs to the PI3K family and regulates an array of cellular functions, including protein synthesis, metabolism, and cell proliferation. It is found in 2 discrete complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which each contain distinct protein components and phosphorylate different substrates. The mTORC1 is acutely inhibited by rapamycin, which has been used therapeutically and experimentally as a probe to gain insight into mTORC1 regulation and function.[8] The mTORC2 is significantly less sensitive to rapamycin than mTORC1.[9] mTORC1 is known to suppress autophagy, which is a key catabolic process.[10] The mTOR is phosphorylated by Akt1 at Ser2448 to yield phospho-mTOR (p-mTOR), which regulates ribosome biogenesis and protein synthesis,[11] and is also present in the cytoplasm and nucleus, with high p-mTOR expression indicating reduced response to tumor treatment.[12,13]

Rapamycin is a potent and specific mTOR inhibitor.[14,15] The mTOR signaling pathway can also be suppressed by genes, such as tuberous sclerosis complex 2 (*TSC2*)[16,17] and phosphatase and tensin homolog deleted on chromosome ten (*PTEN*).[16] The loss or mutation of these genes leads to a wide variety of human sporadic and inherited cancer. We have observed increased activity of the mTOR pathway after silencing PTEN.[18] In the present study, we knocked down PTEN to activate the mTOR pathway.

Hypoxia-inducible factor-1α (HIF-1α), a downstream target of mTOR, is a major transcription factor that controls cellular adaptation to hypoxia and can influence glycolytic metabolism under not only hypoxic but also normoxic conditions.[19,20] The HIF-1α triggers the expression of glycolytic markers that influence the glycolytic rate in tumor cells. These alterations increase tumor cell proliferation (Warburg effect).[21] Moreover, knocking down HIF-1α inhibits lactate production and pyruvate kinase M2 isoform (PKM2) expression in pancreatic cancer.[22]

In 1956, Otto Warburg discovered that cancer cells tend to convert glucose into lactate to produce energy under oxygenated conditions, which is termed aerobic glycolysis or the Warburg effect.[23] The PKM2 is an important cancer metabolism enzyme that is responsible for the Warburg effect. In highly glycolytic cancers, pyruvate is converted to lactic acid and adenosine triphosphate to fuel rapid cellular proliferation.[24] Enhanced PKM2 expression is frequently observed in both the cytoplasm and the nucleus in various human cancers,[25] such as pancreatic cancer, hepatocellular carcinoma,[26] colorectal cancer,[25] and cervical cancer,[27] and is also important for tumor growth.[27] Sun *et al*[28] reported that mTOR-induced PKM2 expression in mouse kidney tumors is mediated by HIF-1α and c-Myc,[27] which was previously found to interact with an associated regulatory protein of mTOR.[20,29]

We hypothesized that knocking down PTEN will affect mTOR and thus regulate PKM2, inducing cancer cells to convert glucose into lactic acid to produce energy, which may be the major pathway for cellular energy metabolism in ESCC.

Materials and Methods

Study Population

The study was approved by the local Ethics Committee (2018 no 001), and informed consent was obtained from all patients. All procedures involving human specimens were performed with written informed consent according to the Declaration of Helsinki. The ESCC tissues from patients absent any radiation and chemotherapy who had pathologically confirmed disease between January 2004 and November 2016 at The Second Hospital of Shanxi Medical University were eligible for this study. The tumor group comprised 15 male and 15 female patients aged 28 to 75 years.

Concurrently, we selected 30 (15 males and 15 females) nontumoral tissue samples for the control group. The control and tumor group showed no significant differences in terms of age (control group: 60.5 [11.5]; tumor group: 59.2 [7.4], $t = 0.548$, $P = .586$). In the tumor group, tumor staging ranged as follows: I, 8/30 (26.7%); II, 13/30 (43.3%); III, 9/30 (30%); and IV, 0/30 (0%).

Immunohistochemistry Analysis

Tissue blocks were subjected to routine hematoxylin and eosin staining to confirm the diagnosis. Sections from the tissue blocks were sliced at 4-μm thickness, mounted on slides, subjected to antigen retrieval, coated with poly-L-lysine, and treated overnight with the following primary antibodies at 4°C: anti-PKM2 antibody (1:500, PKM2 [D78A4] XP Rabbit mAb #4053, Cell Signaling Technology, USA) and anti-p-mTOR antibody (1:200, Anti-mTOR [phospho-S2448], rabbit polyclonal, Abcam ab84400). The sections were then treated for 1 hour at room temperature with goat antirabbit immunoglobulin G (IgG; H+L) secondary antibody conjugated to horseradish peroxidase (1:1000, Novex A16104SAMPLE; Life Technologies, New Zealand), followed by incubation with ABC peroxidase for 1 hour, staining with diaminobenzidine for 5 minutes and counterstaining with hematoxylin (Gene Tech, China). As a negative control, the primary antibody was replaced with phosphate buffer saline (PBS). All sections were assessed at 200× magnification via light microscopy as follows. We quantitatively scored the tissue sections according to the percentage of positive cells and staining intensity, as described below.[30] We rated the intensity of staining on a scale of 0 to 3: 0, negative; 1, weak; 2, moderate; and 3, strong. We assigned the following proportion scores: 0 if 0% of the tumor cells showed positive staining, 1 if 0% to 1% of the cells were stained, 2 if 2% to 10% were stained, 3 if 11% to 30% were stained, 4 if 31% to 70% were stained, and 5 if 71% to 100% were stained. We then combined the proportion and intensity scores to obtain a total score (range: 0-8) as described previously.[30] The results were assessed by 2 experienced pathologists in a blinded manner.

Cell Culture

The human ESCC cell line KYSE150 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in modified RPMI medium (HyClone[T], SH30809.01; Logon, Utah) supplemented with 10% fetal bovine serum (Gibco 1009141, Australia origin) and 1% penicillin-streptomycin (Solarbio P1400, China). The cells were used for experiments when the cells reached 60% confluency.

Rapamycin Assay and Coimmunofluorescence

KYSE150 cells were incubated with rapamycin (VETEC V900930, Sigma, USA) at 0, 10, 20, 50, or 100 nmol/L for 0,

2, 6, 12, or 24 hours to determine the inhibition of mTOR and p-mTOR by coimmunofluorescence. After the cells were fixed with paraformaldehyde, they were incubated with primary antibodies against mTOR (anti-mTOR antibody [53E11], mouse monoclonal, Abcam ab87540, USA) and p-mTOR (anti-mTOR [phospho-S2448], rabbit polyclonal, Abcam ab84400, USA) followed by treatment with secondary antibodies (donkey anti-mouse IgG [H+L] highly cross-adsorbed secondary antibody, Alexa Fluor 488; donkey anti-rabbit secondary antibody, Alexa Fluor 594; Invitrogen, USA). Fluorescence was observed on an Olympus IX 71 (Olympus, Japan) microscope.

Modification of PKM2 and mTOR Expression

The *PKM2* gene was knocked down based on the target sequence gcctacctgtatgtcaataaa using OmicsLink short hairpin RNA (shRNA) expression clone (FulenGen HSH013185-CH1, GeneCopoeia[T], Guangzhou, China). The *PKM2* gene was overexpressed using OmicsLink expression clone (FulenGen EX-Z7438-M29, GeneCopoeia, NM_002654). Both constructs were transfected into cells using the Lipofectamine 2000 transfection teagent (Invitrogen, 11668027). The *PTEN* gene was also knocked down using OmicsLink shRNA expression clone (FulenGen HSH015535-CH1, GeneCopoeia) with Lipofectamine 2000 transfection reagent. Transfection was conducted for 48 hours, after which successful transfection was defined as at least 70% of the cells exhibiting green fluorescence.

Quantitative polymerase chain reaction (qPCR) was used to confirm the transfection of the appropriate plasmids. Total RNA was extracted from KYSE150 cells using an RNA simple total RNA kit (TIANGEN, DP419, China) according to the manufacturer's instructions and then reverse-transcribed and subjected to real-time (RT) PCR analysis to examine PKM2 and PTEN expression levels. The reverse transcription reactions were performed on a Takara RR047A, whereas the qPCR reactions were performed on a Takara RR820A (One-Step SYBR PrimeScript RT-PCR kit) using the StepOnePlus qPCR system (Thermo Fisher, USA) and the following primers:

GAPDH: FP, 5′-ACAACTTTGGTATCGTGGAAGG-3′ and RP, 5′-GCCATCACGCCACAGTTTC-3′; PKM2: FP, 5′-TCGGGCTGAAGGCAGTGATGTG-3′ and RP, 5′-AGGCGG CGGAGTTCCTCAAATA-3′; PTEN: FP, 5′-GAGCGTGCA-GATAATGACAAGG-3′ and RP, 5′-GGATTTGACGGCTC CTCTACTG-3′.

Data showed the target sequence gcctacctgtatgtcaataaa for PKM2 shRNA and the target sequence ggcgtatacaggaacaatatt for PTEN shRNA.

Western Blotting

The cells were washed with PBS, then lysed in RIPA buffer containing protease inhibitors 1% phenylmethylsulfonyl fluoride (PMSF) . Proteins were harvested by centrifugation and then 45 μg protein from different samples were separated by 8% sodium dodecyl sulfate-polyacrylamide gels (Solarbio P1200) electrophoresis and then transferred to polyvinylidene
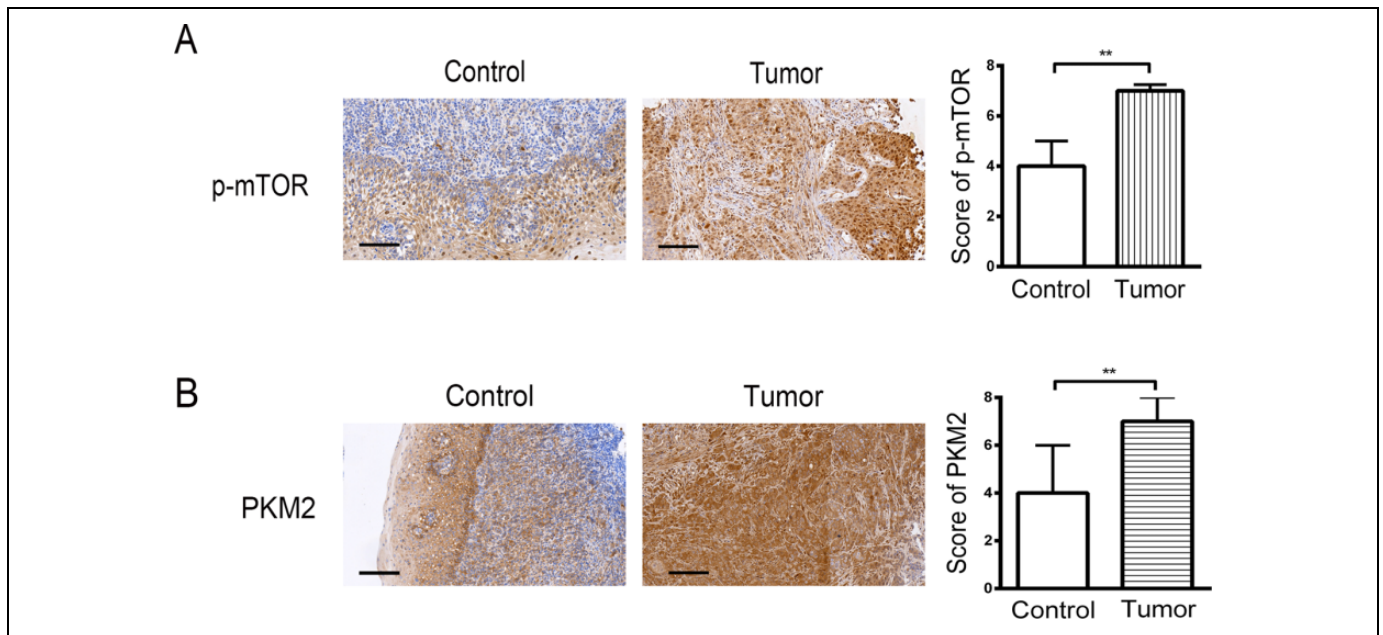
Figure 1. p-mTOR and PKM2 expression scores in the tumor group are higher than control group. A, p-mTOR antibody-stained nontumoral tissues and tumor tissues (magnification, ×200). p-mTOR shows high score in tumor tissues than in nontumoral tissues. B, PKM2 antibody-stained nontumoral tissues and tumor tissues (magnification, ×200). Tumor tissues show high score than nontumoral tissues. The bar plots show the score of the antibody-stained areas that were stained with the same antibody in nontumoral tissues and tumor tissues. Values are means (SD; n = 30, ** $P < .01$). mTOR indicates mammalian target of rapamycin; PKM2, pyruvate kinase M2 isoform; p-mTOR, phospho-mTOR; SD, standard deviation.

fluoride membranes using a Bio-Rad wet-transfer apparatus. The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline/0.1% Tween 20 for 1 hour at room temperature and then incubated overnight at 4°C with the primary antibodies. Immunoblot analysis was performed using secondary antibodies conjugated to horseradish peroxidase, and the primary antibodies included anti-mTOR, anti-p-mTOR, anti-PKM2, and anti-GAPDH (Sangon Biotech, D110016, China). The blots were visualized using a Bio-Rad (Bio-Rad, USA) Western blot detection system.

Lactate Assay

Lactic acid from the culture media of KYSE150 cells in (1) the PKM2-downregulation group, (2) the PKM2-overexpression group, (3) the PTEN-regulation group (as an mTOR activation group), and (4) the rapamycin group was measured using a BioVision lactate colorimetric assay kit II (#K627-100) and a Spectra-Max M2 MultiMode microplate reader (Molecular Devices, Sunnyvale, California) at 450 nm.

Statistical Analysis

Unless otherwise stated, the results presented as the mean (standard deviation) of at least 3 independent experiments. Statistical significance was measured using Student t test, where $P < .01$ was deemed significant. An appropriate posttest has been applied for internal comparisons. All data were analyzed using SPSS 22.0 (SPSS, Inc, Chicago).
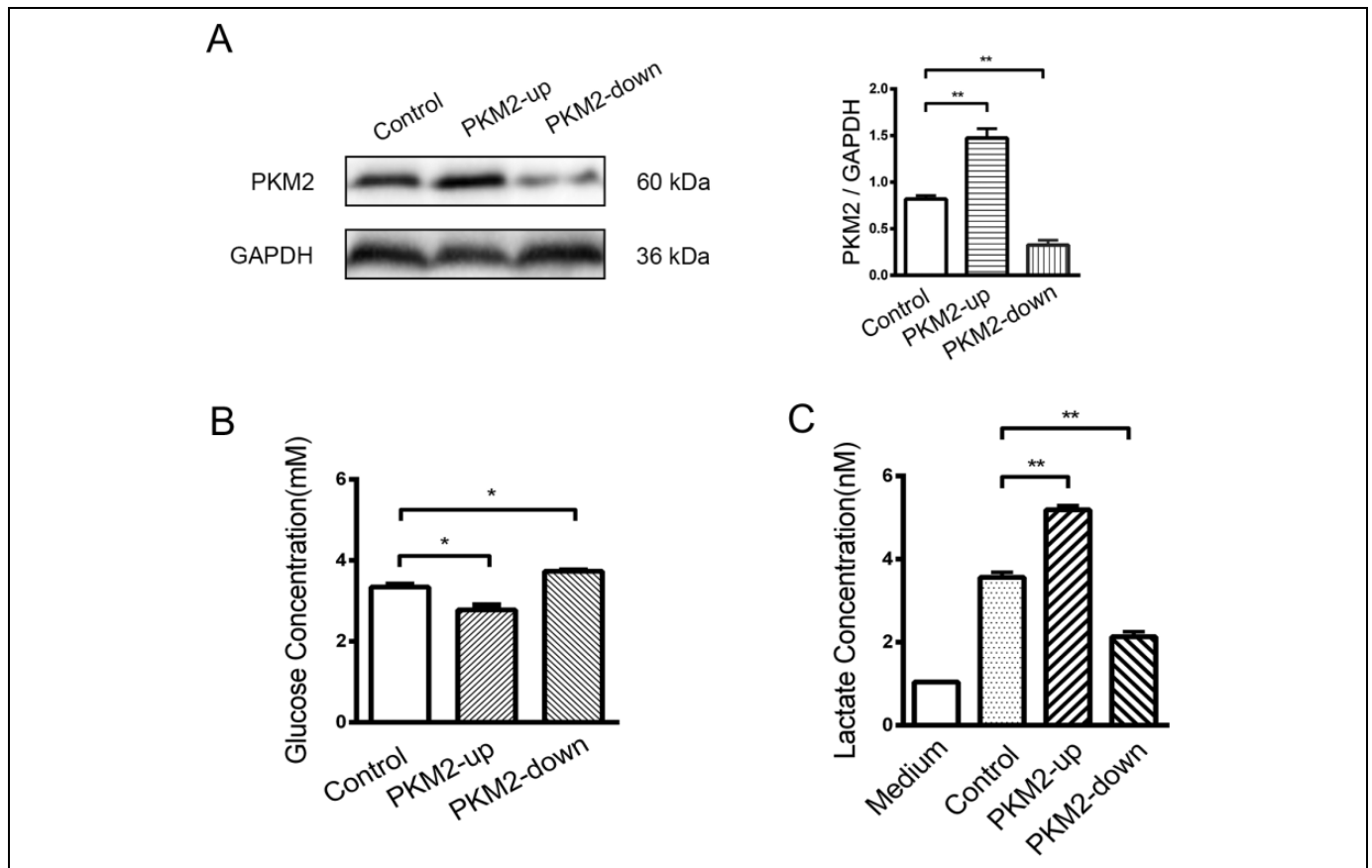
Results

Both PKM2 and mTOR Are More Highly Expressed in Samples From Patients With ESCC

Immunohistochemical analyses (Figure 1) showed that the p-mTOR expression score in the tumor group was 7.00 (0.25) in the cytoplasm and nucleus, which was significantly higher than that in the control group (4.00 [1.00], μ = 6.73, $P < .01$). Similarly, PKM2 expression in the tumor group was 7.00 (1.00) in the cytoplasm and nucleus compared to 4.00 (2.00) in the control group (μ = 6.81, $P < .01$). Thus, both p-mTOR and PKM2 are overexpressed in tumor tissues, which have important implications in the regulation of glycolysis in ESCC.

The control and tumor groups did not show a normal distribution and were described as the median and interquartile range, and the Wilcoxon rank-sum test was used to determine statistical significance. The positive rates of Bartonella in different areas and genders were analyzed using the χ^2 test. $P < .01$ was considered statistically significant (Figure 1).

Modification of PKM2 Regulates Metabolism in an ESCC Cell Line

In the KYSE150 ESCC cell line, the *PKM2* gene was overexpressed and knocked down in different groups using shRNA. Western blotting confirmed the modification of PKM2 expression (Figure 2A). The remaining glucose in the medium showed that the PKM2-up and PKM2-down groups consumed

Figure 2. PKM2 affects glycolysis in ESCC. A, Western blot of PKM2. Overexpression and knockdown of the *PKM2* gene using shRNA resulted in high and low levels, respectively, of PKM2 protein. PKM2 expression was measured by Western blotting. GAPDH was used as a control for protein loading and integrity. B, Glucose assay. The remaining medium of the PKM2-up group contained less glucose and that of the PKM2-down group contained more glucose than the control group. C, Lactate assay. The remaining medium of the PKM2-up group produced more lactate and that of the PKM2-down group produced less lactate than the control group. Data are presented as the means (SD) of 3 independent measurements (n = 3, $* P < .05$, $**P < .01$). PKM2 indicates pyruvate kinase M2 isoform; ESCC, esophageal squamous cell carcinoma; shRNA, short hairpin RNA; GAPDH; SD, standard deviation.

more and less glucose, respectively, than the control group (Figure 2B). Lactate assays showed that modifying PKM2 expression also affected lactate production. The PKM2-up group produced more lactate and the PKM2-down group produced less lactate than the control group (Figure 2C). These results indicated that a high level of PKM2 increased glycolysis, whereas a low level of PKM2 inhibited glycolysis.
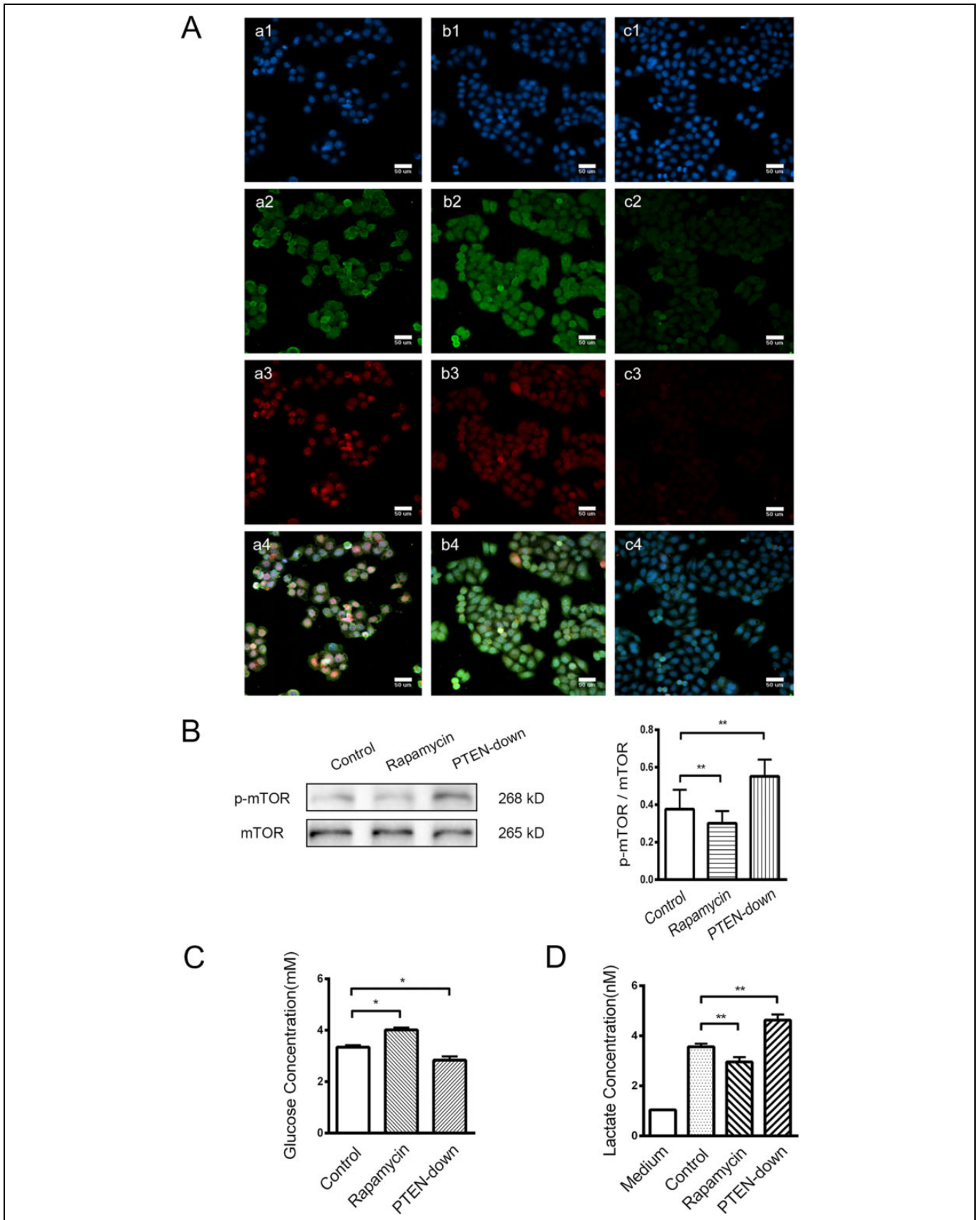
The mTOR Pathway Regulates Metabolism in an ESCC Cell Line

As shown in Figure 3A, the rapamycin-treated group exhibited downregulation of mTOR. Coimmunofluorescence assays showed that in the cells treated with 20 or 50 nmol/L rapamycin for 12 hours, p-mTOR was inhibited. We selected the cells incubated with 50 nmol/L rapamycin as the rapamycin group, aiming to inhibit mTOR and p-mTOR expression. Western blotting revealed that rapamycin inhibited p-mTOR protein levels (Figure 3B).

Measurement of the remaining glucose in the medium showed that the rapamycin group consumed less glucose than did the control group (Figure 3C). Furthermore, in the lactate assay, the rapamycin group produced less lactate than did the control group (Figure 3D). These results indicate that inhibition of the mTOR pathway resulted in the generation of less lactate and that the mTOR pathway regulates glycolysis.

The amino acid sequence of mTOR is too long to be overexpressed in ESCC cells such as KYSE150. However, the *PTEN* gene is a key suppressor gene in the mTOR pathway, as mentioned earlier; therefore, we knocked down PTEN to activate mTOR. The *PTEN* gene was knocked down using shRNA to activate the mTOR pathway, and the modified PTEN expression was confirmed by qPCR. Western blotting showed that knocking down PTEN was associated with increased p-mTOR expression (Figure 3B).

The remaining glucose in the medium showed that the PTEN-down group consumed more glucose than did the control group (Figure 3C), and in the lactate assay, the PTEN-down group produced more lactate than did the control group

Figure 3. mTOR regulates metabolism in an ESCC cell line. A, Fluorescence staining of KYSE150 cells treated with rapamycin. a1, a2, a3, a4: control group; b1, b2, b3, b4: rapamycin-treated cells (20 nM) after 12 hours; c1, c2, c3, c4: rapamycin-treated cells (50 nM) after 12 hours. Blue fluorescence shows the cell nucleus, green fluorescence indicates mTOR, and red fluorescence indicates p-mTOR. B, Western blot of p-mTOR.

(Figure 3D). These results indicated that removal of PTEN inhibition activated the mTOR pathway and generated more lactate. Taken together, the results show that the mTOR pathway regulates metabolism and glycolysis.

The mTOR Pathway Regulates PKM2 to Affect Metabolism

Compared to the control group, PKM2 levels in the rapamycin group were downregulated (Figure 4A) and those in the PTEN-down group were upregulated (Figure 4A). These results showed that PKM2 was inhibited when the mTOR pathway was inhibited, whereas PKM2 was overexpressed when the mTOR pathway was activated. The glucose and lactate assay results shown in Figures 2 and 3 indicate that activation of the mTOR pathway had a similar effect on glycolysis as that of PKM2 overexpression.

We knocked down both PTEN and PKM2 in the same group (PTEN-down + PKM2-down group) to activate mTOR and inhibit PKM2. The glucose and lactate assays showed that glucose consumption (Figure 4B) and lactate production (Figure 4C) did not differ between this group and the PKM2-down group, indicating that activation of mTOR cannot increase aerobic glycolysis if PKM2 is knocked down in ESCC.

We found that activating mTOR regulates PKM2 to induce the Warburg effect in KYSE150 cells. Inhibitors of mTOR (including rapamycin derivatives) and PKM2 are thus potential drugs that could serve as new therapeutic options for cancer treatment (Figure 4B).

Discussion

PKM2 acts as a key enzyme for aerobic glycolysis. The mTOR pathway also plays a crucial role in tumors. In our study, we provided the first evidence that PKM2 is regulated by the mTOR pathway in ESCC. First, PKM2 and mTOR were both overexpressed in ESCC tissues, as determined by immunohistochemical analysis. Second, the results of glycolysis tests after alteration in PKM2 and mTOR expression confirm that the mTOR pathway regulated PKM2 to affect glycolysis in ESCC. These studies confirm the correlation between PKM2 and mTOR and indicate the possible mechanism underlying their relationship, which is crucial to affect glycolysis in ESCC.

PKM2 and mTOR are both important signaling molecules in cancer.[28] Sun et al found that the mTOR pathway was able to affect PKM2 in human pancreatic (PANC-1), prostate (PC3), and liver (HepG2) cancer cell lines.[28] Our immunohistochemical staining data suggest that PKM2 and p-mTOR are both overexpressed in ESCC tissues relative to their expression

levels in nontumoral tissues. mTOR is phosphorylated by Akt1 at Ser2448, leading to downstream signaling by p-mTOR.[11] The premise of our study was to examine the regulatory relationship between the mTOR pathway and PKM2.

PKM2 is considered an important enzyme that converts phosphoenolpyruvate to pyruvate in glycolysis.[31] Lactate is one of the main products of cell aerobic glycolysis, especially in cancer, and glucose is metabolized to lactate through glycolysis.[32,33] We suggested that the consumption of glucose and the production of lactate reflect the efficiency of cancer aerobic glycolysis. We knocked down or overexpressed the *PKM2* gene using OmicsLink™ shRNA. The consumption of glucose and the production of lactic acid by ESCC cells changed following the modification of PKM2 expression. Gu et al and David et al found similar trends in other cancers.[34,35] The results indicate that the regulation of PKM2 can induce changes in aerobic glycolysis.

The mTOR signaling pathway plays a central role in a wide spectrum of cellular activities, including cell proliferation, survival, and differentiation.[36] This pathway also acts as a cellular rheostat that integrates the signals from a variety of cellular signaling pathways to sense growth factors, nutrient availability, and energy status.[37] Studies by Sun et al showed that PKM2 is an important glycolytic enzyme in the oncogenic mTOR-induced Warburg effect. The HIF-1α and c-Myc-hnRNP cascades are the transducers of mTOR regulation of PKM2 in mouse kidney tumors.[28] Zhou et al showed that pharmacological inhibition of mTOR signaling with rapamycin decreased the glycolytic capacity of 6 hepatocellular carcinoma cell lines.[38] mTOR is now recognized as one of the major molecules in the pathway responsible for chemoresistance,[36,39] which is activated in several oncogenic signaling pathways and can be activated by many molecules in cancer.[40] We thus knocked down PTEN to activate mTOR and inhibited mTOR with rapamycin. Western blotting showed that HIF-1α changes according to mTOR expression. We performed tissue sectioning and Western blotting, which indicated that PKM2 also changes according to the expression of mTOR. This result suggests that the mTOR pathway regulates the expression of PKM2 in ESCC. The glucose assay and lactate assay showed that glycolysis changed with the level of mTOR and the regulation of PKM2. We conclude that the mTOR signaling pathway can regulate PKM2 in aerobic glycolysis to affect ESCC cells.

The PKM2 and mTOR pathways are known to be important signaling nodes, and drugs related to these pathways are undergoing laboratory tests and clinical trials for cancer treatment.[41] Tang et al found that metformin was able to inhibit PKM2 through the mTOR pathway, indicating that PKM2 is an important signal molecule.[42] As described above, PKM2 is not only

Figure 3. (Continued). p-mTOR was inhibited by rapamycin. p-mTOR was activated by downregulation of PTEN. mTOR served as a control for protein loading and integrity. C, Glucose assay. The remaining medium of the rapamycin group contained more glucose than did that of the control group, whereas that of the PTEN-down group contained less glucose. D, Lactate assay. The rapamycin group produced less lactate and the PTEN-down group produced more lactate compared with the control group. Data are presented as the means (SD) of 3 independent measurements (n = 3, *$P < .05$, ** $P < .01$). mTOR indicates mammalian target of rapamycin; ESCC, esophageal squamous cell carcinoma; p-mTOR, phospho-mTOR; PTEN, phosphatase and tensin homolog deleted on chromosome ten; SD, standard deviation
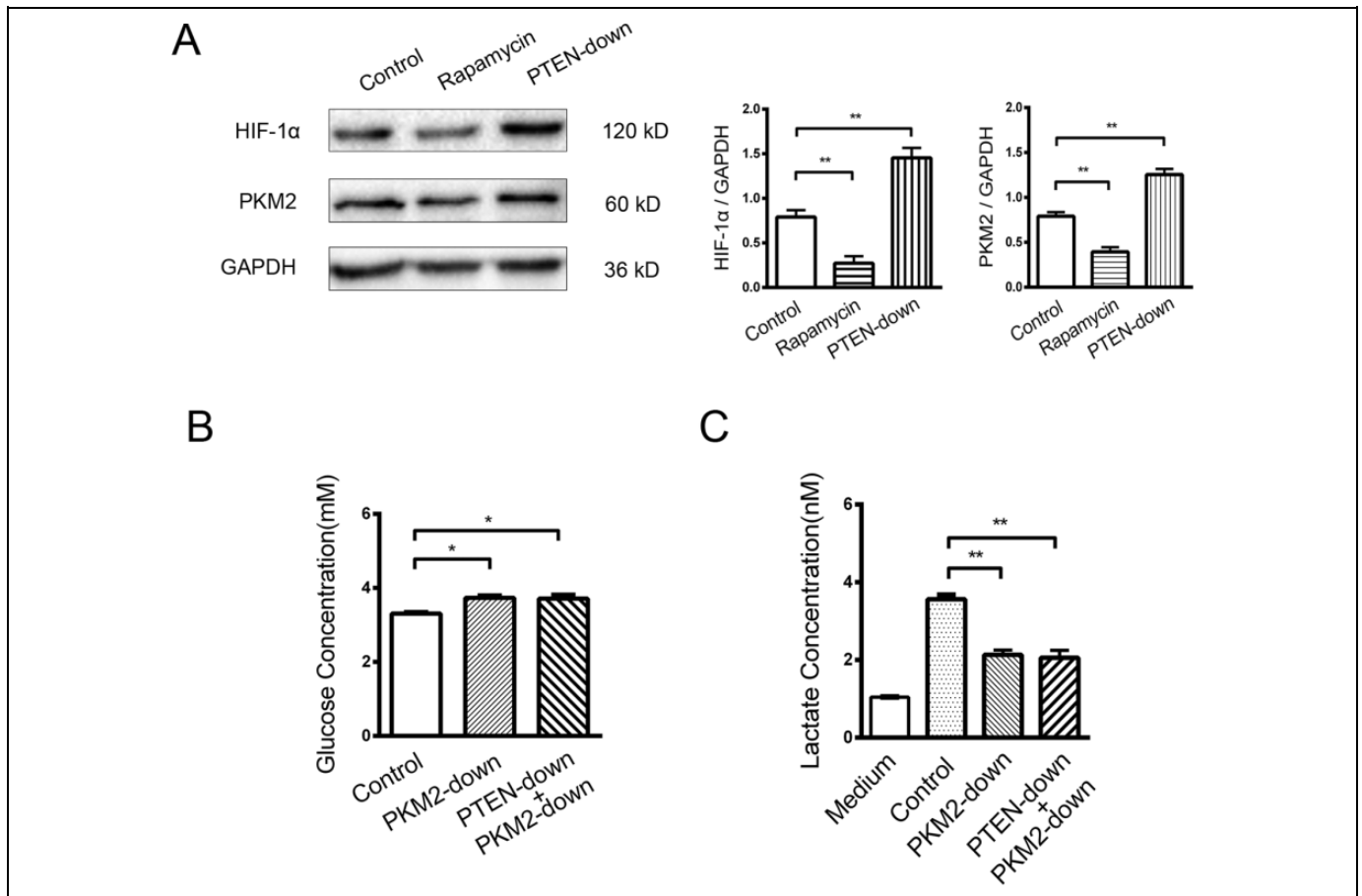
Figure 4. The mTOR pathway regulates PKM2 expression. A, Western blot of PKM2. PKM2, and HIF-1α are downregulated in the rapamycin group and upregulated in the PTEN-down group, as measured by Western blotting. Glyceraldehyde phosphate dehydrogenase (GAPDH) served as a control for protein loading and integrity. B, Glucose assay. The PTEN-down + PKM2-down group showed the same results as the PKM2-down group in the glucose assay. C, Lactate assay. The PTEN-down + PKM2-down group showed the same results as did the PKM2-down group in lactate assays. Student *t* test; data are presented as the means (SD) of 3 independent measurements (n = 3, * $P < .05$, ** $P < .01$). mTOR indicates mammalian target of rapamycin; PKM2, pyruvate kinase M2 isoform; HIF-1α, hypoxia-inducible factor-1α; PTEN, phosphatase and tensin homolog deleted on chromosome ten; GAPDH; SD, standard deviation.

associated with disease progression and invasion but also involved in glycolysis.[26] Drugs that inhibit enzyme sensitivity to substrates could also be considered for treatment. New therapies are needed to treat tumors, which are still a crucial health issue in the world. The mechanism of aerobic glycolysis draws our attention once again. Our experiments prove that mTOR can regulate PKM2 in ESCC, forming the basis for further exploring aerobic glycolysis, including through animal experiments *in vivo*. In addition, survival time and individualized therapy plans should be taken into consideration. Our research may provide new insights into cancer treatment and suggests that a multitarget approach be applied to obtain multiple benefits.

Authors' Note

He X, Ma J, Shi S, and Cheng K conceived and designed the experiments. He X and Yin performed the biochemical experiments. He X and Yin analyzed the data. Yan Z, Cheng S, Wang Y, Cheng D, and Bai X collected clinical information and tissue samples. Zhang W performed the immunohistochemical staining assay. He X wrote the article.

Declaration of Conflicting Interests

Funding

Ethical Approval

The study was approved by The Shanxi Medical University Ethics Committee (2018 no 001), and informed consent was obtained from all the patients. All of the procedures involving human specimens were performed with written informed consent according to the Declaration of Helsinki.

References

1. Di Pardo BJ, Bronson NW, Diggs BS, Thomas CR Jr, Hunter JG, Dolan JP. The global burden of esophageal cancer: a disability-adjusted life-year approach. *World J Surg*. 2016; 40(2):395-401.

2. Chen W, Zheng R, Baade PD, et al. Cancer statistics in China, 2015. *CA Cancer J Clin*. 2016;66(2):115-132.

3. Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *J Clin Oncol*. 2006;24(14): 2137-2150.

4. Fu HL, Wu DP, Wang XF, et al. Altered miRNA expression is associated with differentiation, invasion, and metastasis of esophageal squamous cell carcinoma (ESCC) in patients from Huaian, China. *Cell Biochem. Biophys*. 2013;67(2):657-668.

5. Wang GQ, Jiao GG, Chang FB, et al. Long-term results of operation for 420 patients with early squamous cell esophageal carcinoma discovered by screening. *Ann Thorac Surg*. 2004;77(5): 1740-1744.

6. Xie J, Wang X, Proud CG. mTOR inhibitors in cancer therapy. *F1000Res*. 2016;5. doi:10.12688/f1000research.9207.1

7. Vanhaesebroeck BL, Stephens L, Hawkins P. PI3K signalling: the path to discovery and understanding. *Nat Rev Mol Cell Biol*. 2012; 13(3):195-203.

8. Kennedy BK, Lamming DW. The mechanistic target of rapamycin: the grand conducTOR of metabolism and aging. *Cell Metab*. 2016;23(6):990-1003.

9. Sarbassov DD, Ali SM, Sengupta S, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell*. 2006;22(2):159-168.

10. Kim YC, Guan KL. mTOR: a pharmacologic target for autophagy regulation. *J. Clin. Invest*. 2015;125(1):25-32.

11. Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N. mTOR, translation initiation and cancer. *Oncogene*. 2006;25(48): 6416-6422.

12. Bostner J, Karlsson E, Pandiyan MJ, et al. Activation of Akt, mTOR, and the estrogen receptor as a signature to predict tamoxifen treatment benefit. *Breast Cancer Res Treat*. 2013;137(2): 397-406.

13. Zhai XX, Tang ZM, Ding JC, Lu XL, et al. Expression of TGF-beta1/mTOR signaling pathway in pathological scar fibroblasts. *Mol Med Rep*. 2017;15(6):3467-3472.

14. Deblon N, Bourgoin L, Veyrat-Durebex C, et al. Chronic mTOR inhibition by rapamycin induces muscle insulin resistance despite weight loss in rats. *Br J Pharmacol*. 2012. 165(7):2325-2340.

15. Tsang CK, Qi H, Liu LF, Zheng XF. Targeting mammalian target of rapamycin (mTOR) for health and diseases. *Drug Discov Today*. 2007;12(3-4):112-124.

16. Nathan N, Keppler-Noreuil KM, Biesecker LG, Moss J, Darling TN. Mosaic disorders of the PI3K/PTEN/AKT/TSC/mTORC1 signaling pathway. *Dermatol Clin*. 2017;35(1):51-60.

17. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol*, 2002;4(9):648-657.

18. Podsypanina K, Lee RT, Politis C, et al. An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten$^{+/-}$ mice. *Proc Natl Acad Sci U S A*. 2001;98(18):10320-10325.

19. Semenza G.L. HIF-1 mediates the Warburg effect in clear cell renal carcinoma. *J. Bioenerg Biomembr*. 2007;39(3): 231-234.

20. Land SC, Tee AR. Hypoxia-inducible factor 1alpha is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. *J Biol Chem*. 2007;282(28):20534-20543.

21. Moench R, Grimmig T, Kannen V, et al. Exclusive inhibition of PI3K/Akt/mTOR signaling is not sufficient to prevent PDGF-mediated effects on glycolysis and proliferation in colorectal cancer. *Oncotarget*. 2016;7(42):68749-68767.

22. He G, Jiang Y, Zhang B, Wu G. The effect of HIF-1alpha on glucose metabolism, growth and apoptosis of pancreatic cancerous cells. *Asia Pac J Clin Nutr*. 2014;23(1):174-180.

23. Lu CL, Qin L, Liu HC, Candas D, Fan M, Li JJ. Tumor cells switch to mitochondrial oxidative phosphorylation under radiation via mTOR-mediated hexokinase II inhibition – a Warburg-reversing effect. *PLoS One*. 2015;10(3):e0121046.

24. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009;324(5930):1029-1033.

25. Ginés A, Bystrup S, Ruiz de Porras V, et al. PKM2 subcellular localization is involved in oxaliplatin resistance acquisition in ht29 human colorectal cancer cell lines. *PLoS One*. 2015;10(5):e0123830.

26. Fan F, Wu H, Liu Z, et al. Nuclear PKM2 expression, an independent risk factor for ER after curative resection of hepatocellular carcinoma. *Biomed Pharmacother*. 2016;84:1858-1864.

27. Yuan Y, Guo-Qing P, Yan T, Hong-Lin Y, Gong-Hua H, Cai-Gao Z. A study of PKM2, PFK-1, and ANT1 expressions in cervical biopsy tissues in China. *Med Oncol*. 2012;29(4):2904-2910.

28. Sun Q, Chen X, Ma J, et al. Mammalian target of rapamycin up-regulation of pyruvate kinase isoenzyme type M2 is critical for aerobic glycolysis and tumor growth. *Proc Natl Acad Sci U S A*. 2011;108(10):4129-4134.

29. Dang CV, Kim JW, Gao P, Yustein J. The interplay between MYC and HIF in cancer. *Nat Rev Cancer*. 2008;8(1):51-56.

30. Liang J, Cao R, Wang X, et al. Mitochondrial PKM2 regulates oxidative stress-induced apoptosis by stabilizing Bcl2. *Cell Res*. 2017;27(3):329-351.

31. Lu Z. Nonmetabolic functions of pyruvate kinase isoform M2 in controlling cell cycle progression and tumorigenesis. *Chin J Cancer*. 2012;31(1):5-7.

32. Ferreira LM. Cancer metabolism: the Warburg effect today. *Exp Mol Pathol*. 2010;89(3):372-380.

33. Bensinger SJ, Christofk HR. New aspects of the Warburg effect in cancer cell biology. *Semin Cell Dev Biol*. 2012;23(4):352-361.

34. Gu Z, Xia J, Xu H, Frech I, Tricot G, Zhan F. NEK2 promotes aerobic glycolysis in multiple myeloma through regulating splicing of pyruvate kinase. *J Hematol Oncol*. 2017;10(1):17.

35. David CJ, Chen M, Assanah M, Canoll P, Manley JL. HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature*. 2010;463(7279):364-368.

36. Xie S, Chen M, Yan B, He X, Chen X, Li D. Identification of a role for the PI3K/AKT/mTOR signaling pathway in innate immune cells. *PLoS One*. 2014;9(4):e94496.

37. Zhang L, Wu J, Ling MT, Zhao L, Zhao KN. The role of the PI3K/Akt/mTOR signalling pathway in human cancers induced by infection with human papillomaviruses. *Mol Cancer*. 2015;14:87.

38. Zhao X, Jiang P, Deng X, et al. Inhibition of mTORC1 signaling sensitizes hepatocellular carcinoma cells to glycolytic stress. *Am J Cancer Res*. 2016;6(10):2289-2298.

39. Wu C, Wangpaichitr M, Feun L, et al. Overcoming cisplatin resistance by mTOR inhibitor in lung cancer. *Mol Cancer*. 2005;4(1):25.

40. Guridi M, Kupr B, Romanino K, et al. Alterations to mTORC1 signaling in the skeletal muscle differentially affect whole-body metabolism. *Skelet Muscle*. 2016;6(1):13.

41. Yang A, Xiao X, Zhao M, LaRue AC, Schulte BA, Wang GY. Differential reponses of hematopoietic stem and progenitor cells to mTOR inhibition. *Stem Cells Int*. 2015;2015:561404.

42. Tang JC, An R, Jiang YQ, Yang J. Effects and mechanisms of metformin on the proliferation of esophageal cancer cells in vitro and in vivo. *Cancer Res Treat*. 2016;43(9):778-789.