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PKM2 Thr454 phosphorylation increases its nuclear translocation and promotes xenograft tumor growth in A549 human lung cancer cells

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A B S T R A C T

Pyruvate kinase M2 (PKM2) is a key enzyme of glycolysis which is highly expressed in many tumor cells, and plays an important role in the Warburg effect. In previous study, we found PIM2 phosphorylates PKM2 at Thr454 residue (Yu, etl 2013). However, the functions of PKM2 Thr454 modification in cancer cells still remain unclear. Here we find PKM2 translocates into the nucleus after Thr454 phosphorylation. Replacement of wild type PKM2 with a mutant (T454A) enhances mitochondrial respiration, decreases pentose phosphate pathway, and enhances chemosensitivity in A549 cells. In addition, the mutant (T454A) PKM2 reduces xenograft tumor growth in nude mice. These findings demonstrate that PKM2 T454 phosphorylation is a potential therapeutic target in lung cancer.

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1. Introduction

Many kinds of cancer cells like to use glucose and produce lactate, exception of oxygen availability, which is known as aerobic glycolysis or the Warburg effect, tumor cell growth also depends on glycolysis [1,2]. Pyruvate kinase (PK) as a rate-limiting enzyme plays at important role in glycolytic pathway [3]. PK catalyzes phosphoenolpyruvic acid into pyruvate, and phosphorylates adenosine diphosphate (ADP) to adenosine triphosphate (ATP) [4]. Mammalian PK has four isoenzymes (PKM1, PKM2, PKL and PKR) which are expressed in different cell types [5]. PKM2 forms an active tetramer which determines glucose is converted to CO_2 and ATP for energy generation, and a relatively inactive dimer or monomer used for the synthesis of cell building in tumor cells which has a high level in tumor cells [6]. Replacing PKM2 with PKM1 in tumor cells diminishes Warburg effect and inhibits tumor formation [7]. However, the mechanisms of PKM2 post-translational modification in Warburg effect remain unclear.

Work from our lab and others demonstrated that PKM2 was regulated by other proteins through direct interaction and modification. Fibroblast growth factor receptor 1 phosphorylates PKM2 on Tyr105, and inhibits its formation of tetramer, but protein tyrosine phosphatase 1B reverses this phosphorylation [8,9]. In addition, acetylation of PKM2 at Lys305 promotes its degradation via chaperone mediated autophagy [10]. Moreover, ERK1/2 has been shown to phosphorylate PKM2 on Ser37 and promotes its nuclear translocation, which is important for cancer cells proliferation [11]. In our previous wok, we found Hsp40 as a novel binding partner of PKM2 destabilized PKM2 protein through HSC70 which inhibited cancer cells glycolysis and proliferation [12]. Moreover, we recently reported PIM2 as a Thr/Ser kinase phosphorylated PKM2 at Thr454 site which increased its protein stability and co-activator functions in cancer cells [13].

In this study, we further demonstrate that PKM2 translocates into the nucleus after Thr454 phosphorylation which is important for its nuclear co-activator function. Our data demonstrate PKM2 Thr454 phosphorylation also promotes xenograft tumor growth in vitro and in vivo, which reveals the association between Warburg effect and tumorigenesis. Our findings provide new insights into the mechanisms underlying PKM2 regulation by T454 phosphorylation in nude mice, which may be a potential therapy target in lung cancer.

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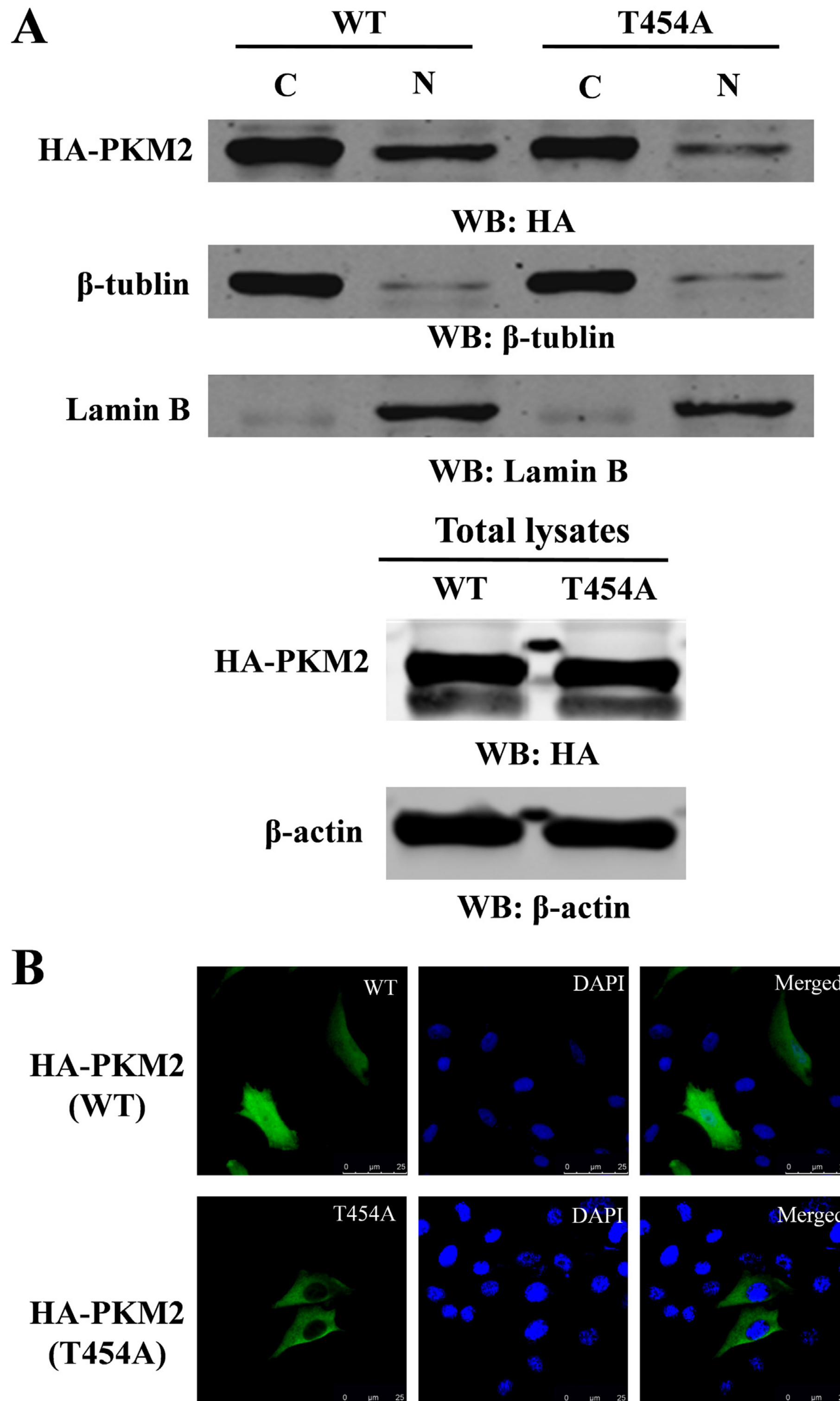

Fig. 1. PKM2 translocates into the nucleus after Thr454 phosphorylation. A. Cytoplasm and nuclear extractions were separated from over-expression HA-PKM2 (WT or T454A) A549 cells followed by western blotting using anti-HA antibody, anti-β-tublin antibody, anti-Lamin B antibody. B. Confocal immunofluorescence microscopy was performed to analyze localization of HA-PKM2 (WT or T454A) in A549 cells.

2. Materials and method

2.1. Antibody

Anti-PKM2 (Abcam), anti-HA (Abmart), anti-β-actin (Sigma), anti-β-tublin (Sigma), anti-Lamin B (Upstate) Goat anti-Mouse second antibody IRDye 800CW (LI-COR) and Goat anti-Rabbit second antibody IRDye 680RD (LI-COR).

2.2. Cell culture and transient transfection

A549 cells were cultured in DMEM (GIBCO) supplemented with

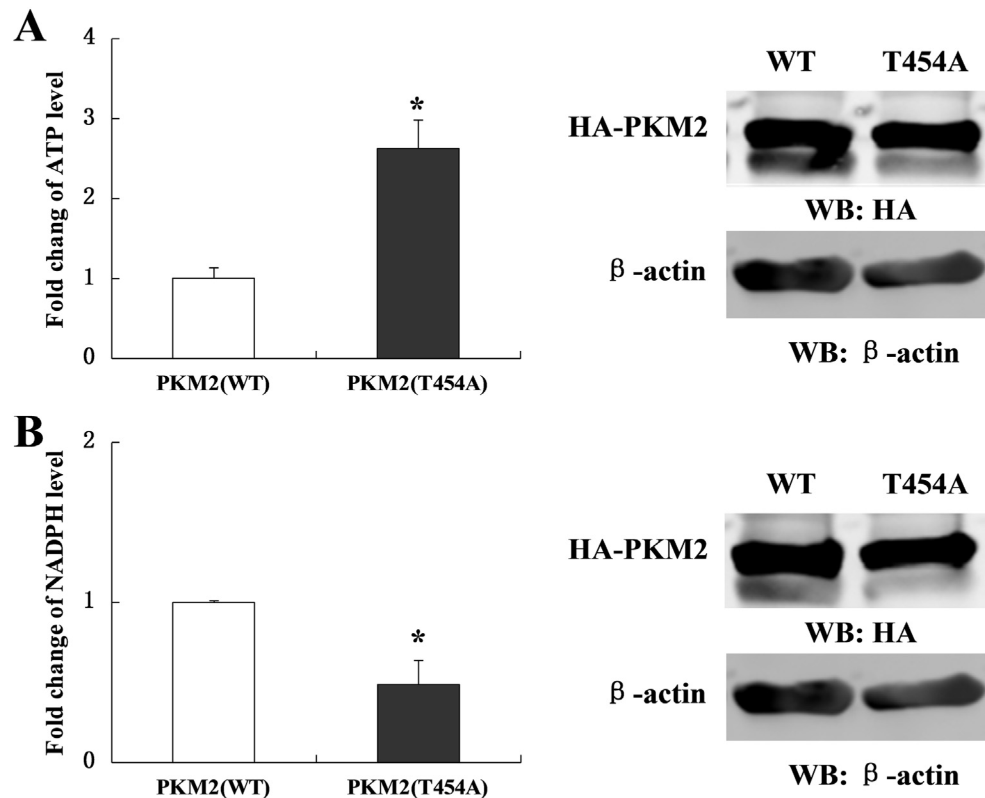

Fig. 2. PKM2 T454 phosphorylation decreases mitochondrion function and enhances pentose phosphate pathway. A. A549 cells were transfected with HA-PKM2 (WT or T454A). Two days after transfection, the cells were collected. ATP levels in cell lysis were examined. (Data represent mean ± SEM n = 3), *p < 0.05. B. A549 cells were transfected with HA-PKM2 (WT or T454A). Two days after transfection, the cells were collected. NADPH levels in cell lysis were examined. (Data represent mean ± SEM n = 3), *p < 0.05.

10% fetal bovine serum (GIBCO), 100 mg/ml penicillin, and 100 mg/ml streptomycin sulfate (Sangon) at 37 °C and 5% CO_2. Lipofectamine 3000 (Invitrogen) was used in transient transfection according to the manufacturer's protocol.

2.3. Subcellular fractionation

Fractionation of nuclear and cytosolic extracts was performed by using NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific) according to the manufacturer's instructions.

2.4. Confocal immunofluorescence microscopy

A549 Cells were plated into 6-well plates with a density of 1×10^5 cells/well. About 48hr after transfection, cells were fixed with 4% paraformaldehyde and treated with 0.2% Triton X-100. The cells were blocked with 3% BSA/PBS for 1hr followed by incubation with primary antibody overnight at 4 °C. Then cells were incubated with a secondary antibody for 1hr and stained with DAPI for 10min. The results were visualized by a confocal laser-scanning microscope (OLYMPUS BX61).

2.5. ATP and NADPH assays

The assays were carried out according to the protocol of ATP Assay (BioVision) and NADP/NADPH Assay Kit (BioVision).

2.6. CCK-8 assay

The assays were carried out according to the protocol of Cell Counting Kit-8 (Dojindo Molecular Technologies). Absorbance was read at 450 nm using an enzyme micro-plate reader. The IC_{50} value was calculated using GraphPad Prism version 5.0 software [14].

2.7. Cell proliferation analysis and xenograft studies

A549 cells were transfected with pcDNA3/HA-PKM2 (WT) or pcDNA3/HA-PKM2 (T454A). After 24hr incubation, we used G418 to screen stable expression A549 cells. 1×10^4 A549 cells were reseeded onto 24-well plates, and cell numbers were counted every 24hr over a four-day period. Nude mice (male 6 to 8 week-old) were injected subcutaneously with 1×10^7 A549 cells. Around 7 weeks after injection, the tumors were dissected and weighed [15,16].

2.8. Statistical analysis

We determined the significance of differences using Pearson's correlation test and Student's t test (two-tailed). P*<0.05 was considered to be significant.

3. Results

3.1. PKM2 translocates into the nucleus after Thr454 phosphorylation

In our previous study, we found PIM2 phosphorylated PKM2 Thr454 site which increased cancer cells glycolysis [13]. PKM2 nuclear translocation plays an important role in regulating cancer cells function [17,18]. So we tested PKM2 nuclear translocation after phosphorylation. We over-expressed HA-PKM2 (WT) and HA-PKM2 (T454A) in A549 cancer cells, and used fluorescence microscopy to test PKM2 nuclear translocation. As showed in Fig. 1B, HA-

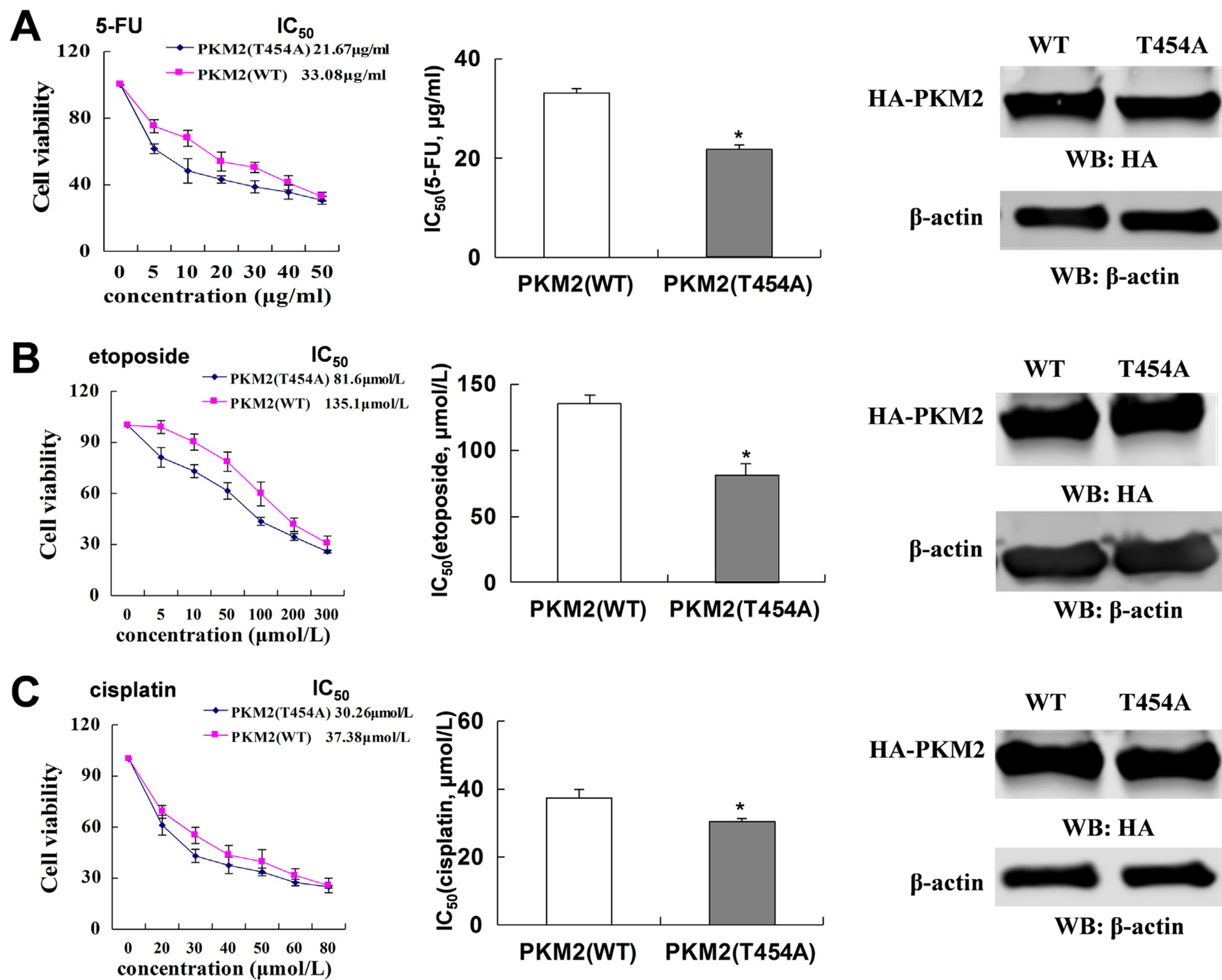

Fig. 3. PKM2 T454 phosphorylation increases resistance to chemotherapy. A, B and C. A549 cells were transfected with HA-PKM2 (WT or T454A). One day after transfection, cells were re-plated in 96-well plates and treated with the indicated drugs. After 24 h, the cell survival rate was examined in CCK-8 assays. The IC_{50} values were calculated using GraphPad Prism version 5.0 software (Data represent mean ± SEM n = 3), $P^{*}<0.05$.

PKM2 after mutating T454A mainly translocated into cytoplasm. To further validate this result, we isolated nucleus and cytoplasm protein from A549 cells. The western blot data showed that PKM2 mainly distributed in the cytoplasm after mutation of PKM2 T454 (Fig. 1A). Together, these data show that PKM2 Thr454 phosphorylation mainly distributed in the nucleus.

3.2. *PKM2 T454 phosphorylation decreases mitochondrion function and enhances pentose phosphate pathway*

PKM2 entered the nucleus after T454 phosphorylation, but PKM2 mainly catalyzed substrates in the cytoplasm. So we supposed that PKM2 T454 phosphorylation reduced mitochondrion function. The ATP levels reflected mitochondrion functions, so we tested ATP levels after transfection of PKM2 (WT or T454A) in A549 cells. As showed in Fig. 2A, PKM2 T454 phosphorylation decreased ATP levels. Pentose phosphate pathway is very crucial for cancer cells proliferation. The NADPH mainly produced from pentose phosphate pathway, which powers redox defense and reductive biosynthesis. PKM2 T454 phosphorylation increased the NADPH levels in A549 cells (Fig. 2B). These findings suggest that PKM2 T454 phosphorylation reduced mitochondrion function and enhanced pentose phosphate pathway.

3.3. *PKM2 T454 phosphorylation increases resistance to chemotherapy*

To determine whether PKM2 T454 phosphorylation affects cancer cell sensitivity to chemotherapeutic drugs, we transfected A549 cells with HA-PKM2 (WT or T454A). As shown in Fig. 3A, compared to wild type, the mutant (T454A) PKM2 appeared to be more sensitive to the chemotherapeutic drug fluorouracil (5-FU)-induced killing with a significant lower IC_{50}. Similarly, over-expression of the mutant (T454A) PKM2 also displayed an increased sensitivity to another two chemotherapeutic drugs etoposide and cisplatin-induced killing in A549 cells (Fig. 3B and C). Thus, our data suggest that PKM2 T454 phosphorylation increases resistance to chemotherapeutic agents.

3.4. *PKM2 T454 phosphorylation promotes cell proliferation and tumor growth*

In previous research, we demonstrated that PKM2 T454 phosphorylation promoted cell proliferation in A549 cells [13]. As

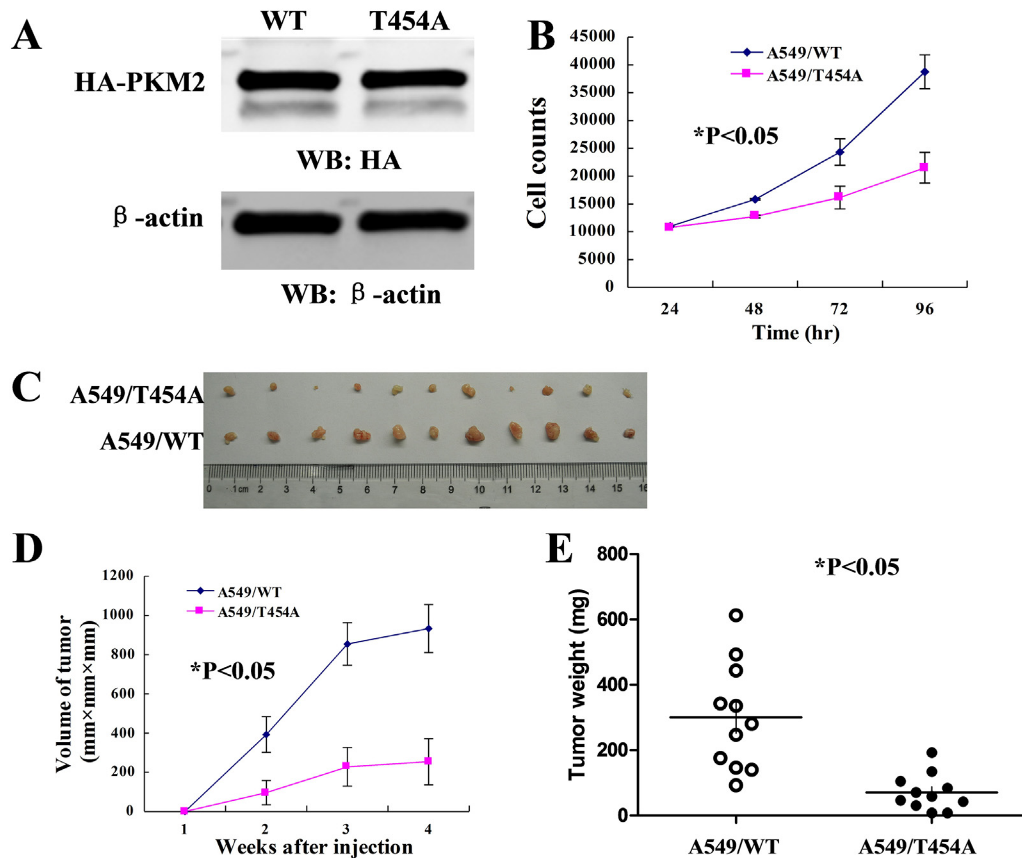

Fig. 4. PKM2 T454 phosphorylation promotes cell proliferation and tumor growth. A. HA-PKM2 (WT or T454A) was stable expressed in A549 cells followed by western blotting using anti-HA antibody. B. HA-PKM2 (WT or T454A) stable expressed A549 cells were planted 1×10^4 cells/well in 48-well culture plate and cell numbers were counted every 24 h for cell proliferation assays. (Data represent mean ± SEM n = 3), $^*p < 0.05$. C, D and E. Nude mice were injected on the left side with A549/WT cells and the right side with A549/T454A cells. The xenograft tumors were measured over time and dissected at the endpoint and shown as A549/WT (lower row) and A549/T454A (upper row) in (C). Quantification of average tumor volume and weight over time is shown in (D and E). Error bars represent ±SD for 11 tumors, $^*p < 0.05$.

shown in Fig. 4A, A549/PKM2 (WT) cells proliferated faster than the A549/PKM2 (T454A) cells. To study whether PKM2 T454 phosphorylation also rendered growth advantage to tumor cells in vivo, we performed xenograft studies. Ten million A549/PKM2 (WT or T454A) cells were injected into nude mice, and tumor cell growth was monitored every week. As shown in Fig. 4B, larger tumors were formed in mice injected with A549/PKM2 (WT) cells than in those injected with A549/PKM2 (T454A) cells. Measurement of the tumor volume and weight demonstrated that A549/PKM2 (WT) cells gave rise to significantly larger tumors than the A549/PKM2 (T454A) cells (Fig. 4C and D). Taken together, these results suggest PKM2 T454 phosphorylation confers tumor cell growth advantage both in vitro and in vivo.

4. Discussion

The Warburg effect was first reported in 1924, and became a typical trait in tumor. Such a characteristic of increased aerobic glycolysis has been used for diagnosis of cancer using ^{18}F-deoxyglucose position emission tomography (PET) [19]. Although it has a widely clinical application, the mechanisms underlying the Warburg effect is still not clear.

In previous study, we demonstrated that PIM2 phosphorylated PKM2 on T454, and caused Warburg effect [13]. Here we find T454 phosphorylation of PKM2 translocates into the nucleus. Nuclear PKM2 has been reported to co-activate HIF-1α, β-catenin and c-MYC which is crucial for tumor cells proliferation and metabolism [20–22]. PKM2 T454 phosphorylation correlates with nuclear translocation which plays a significant role in the progression of these tumors in humans. ATP is the dominant energy source in animals for mechanical and electrical work which is produced from mitochondrion [23]. Our data show that PKM2 T454 phosphorylation decreases ATP production, because aerobic glycolysis has a low efficient to produce ATP compare to mitochondrion. Pentose phosphate pathway produces a lot of metabolic intermediates for biosynthesis to support rapid cell growth, NADPH assays demonstrate that PKM2 T454 phosphorylation enhances pentose phosphate pathway. These data is consistent with tumor cells proliferation.

Chemotherapy is one of the most successful treatments for lung cancer patients, although chemoresistance has become a significant problem for anti-cancer therapy [24]. In this study, we show that PKM2 T454 phosphorylation increases resistance of A549 cells to 5-FU, etoposide and cisplatin, which are common chemotherapeutic agents for lung cancer. Taken together, these observations suggest that therapeutic strategies targeting PKM2 T454 phosphorylation might improve lung cancer sensitivity to chemotherapy.

Previous study indicated that PKM2 Ser37 phosphorylation promoted that PKM2 translocated into the nucleus and co-activated couples of transcriptional factors to increase tumor proliferation in vivo and in vitro [11]. So we select stably transfected cells, and get single clones which have stably expressed HA-PKM2 (WT or T454A). In nude mice, A549/PKM2 (WT) cells grow faster

than A549/PKM2 (T454A) cells. As a result, PKM2 T454 phosphorylation increases tumor cell proliferation in vitro and tumor growth in vivo.

Here we find PKM2 T454 phosphorylation induces PKM2 translocation into the nucleus. Replacement of wild type PKM2 with a mutant (T454A) enhances mitochondrion function, decreases pentose phosphate pathway, and enhances chemosensitivity in lung cancer cells. In addition, the mutant (T454A) PKM2 reduces xenograft tumor growth in nude mice. These findings suggest that PKM2 T454 phosphorylation is a potential therapeutic target in lung cancer. We have thus provided a novel application for targeting PKM2 T454 phosphorylation to overcome lung cancer.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgments

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