Original Articles

Targeting pyruvate kinase M2 contributes to radiosensitivity of non-small cell lung cancer cells in vitro and in vivo

Mao-Bin Meng a,1,*, Huan-Huan Wang a,1, Wen-Hao Guo b,1, Zhi-Qiang Wu a, Xian-Liang Zeng a, Nicholas G. Zaorsky c, Hua-Shan Shi b, Dong Qian a, Zhi-Min Niu a, Bo Jiang a, Lu-Jun Zhao a, Zhi-Yong Yuan a, Ping Wang a

1 Department of Radiation Oncology, CyberKnife Center, and Key Laboratory of Cancer Prevention and Therapy, Tianjin Medical University Cancer Institute & Hospital, National Clinical Research Center for Cancer, Tianjin 300060, China
2 Department of Abdominal Oncology, Cancer Center and State Key Laboratory of Biotherapy, West China Hospital, West China Clinical Medicine School, Sichuan University, Chengdu, Sichuan 610041, China
3 Department of Radiation Oncology, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA

Abstract

Aerobic glycolysis, a metabolic hallmark of cancer, is associated with radioresistance in non-small cell lung cancer (NSCLC). Pyruvate kinase M2 isoform (PKM2), a key regulator of glycolysis, is expressed exclusively in cancers. However, the impact of PKM2 silencing on the radiosensitivity of NSCLC has not been explored. Here, we show a plasmid of shRNA-PKM2 for expressing a short hairpin RNA targeting PKM2 (shRNA-PKM2) and demonstrate that treatment with shRNA-PKM2 effectively inhibits PKM2 expression in NSCLC cell lines and xenografts. Silencing of PKM2 expression enhanced ionizing radiation (IR)-induced apoptosis and autophagy in vitro and in vivo, accompanied by inhibiting AKT and PKD1 phosphorylation, but enhanced ERK and GSK3β phosphorylation. These results demonstrated that knockdown of PKM2 expression enhances the radiosensitivity of NSCLC cell lines and xenografts as well as may aid in the design of new therapies for the treatment of NSCLC.

Introduction

The incidence of lung cancer is increasing, and lung cancer is a leading cause of cancer-related mortality worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancers [2]. Currently, treatment of localized advanced lung cancer includes systemic chemotherapy and radiotherapy [3]. Despite aggressive approaches in the treatment of patients with NSCLC, such as advanced radiotherapy, the efficacy of current therapeutic protocols is limited and the prognosis of patients with NSCLC remains poor. Therefore, it is important to understand the mechanisms by which NSCLC is insensitive to radiotherapy and to develop new strategies for radiotherapies for patients with localized advanced NSCLC. Cancer cells have unique metabolic characteristics of increased glycolysis and lactate production, even in the presence of oxygen, a phenomenon known as aerobic glycolysis or Warburg effect [4]. Aerobic glycolysis is a metabolic hallmark of cancer and is regulated by many factors [5,6]. Previous studies have shown that aerobic glycolysis can generate a chemically reduced milieu associated with the development of radioresistance in cancer cells, and inhibition of glycolysis increases radiosensitivity, thus obtaining a significant therapeutic gain in combination treatments with radiotherapy [7–23]. However, the mechanisms by which inhibition of glycolysis increases radiosensitivity are not fully understood. It is well known that pyruvate kinase M2 isoform (PKM2), one of the four isoforms of pyruvate kinase, occurred in a tetrameric form with a high affinity to its substrate phosphoenolpyruvate (PEP) and a dimeric form with a low affinity to PEP, and the transition between both conformations regulated the glycolytic flux, which is exclusively expressed in cancer-tissue specific [6,24,25]. Furthermore, our previous studies demonstrated that inhibition of PKM2 activity enhances chemotherapeutic drug-inhibited growth of tumors [26,27]. However, whether down-regulation of PKM2 expression could modulate the radiosensitivity of NSCLC cells and how the change in PKM2

Abbreviations: NSCLC, non-small cell lung cancer; PKM2, pyruvate kinase M2 isoform; shRNA, the plasmid encoding a short hairpin RNA; IR, ionizing radiation; PEP, phosphoenolpyruvate; 3-MA, 3-methyladenine; Z-VAD, benzoxycarbonylvalyl-alanyl-aspartyl fluoromethyl ketone; γ-H2AX, phosphorylated histone 2AX; TUNEL, transferase-mediated dUTP nick end labeling; FACS, fluorescence-activated cell sorting; PI, propidium iodide; TEM, transmission electron microscope; LC 3, microtubule-associated protein 1 light chain 3.

* Corresponding author. Tel.: +86 22 23341405; fax: +86 22 23344105.
E-mail address: doctormm991@hotmail.com (M.-B. Meng).

1 These authors contributed equally to this work.
expression could affect the consequence of radiotherapy in NSCLC have not been explored.

In this study, we employed a NSCLC cell lines as a model to test the impact of PKM2 silencing on their radiosensitivity and the potential mechanisms underlying the action of PKM2 silencing in vitro and in vivo. We found that knockdown of PKM2 expression by pshRNA transfection increased the sensitivity of NSCLC cell lines to radiotherapy by enhancing cancer cell apoptosis and autophagy, which was associated with inhibition of AKT and PDK1 phosphorylation, and enhancement of ERK1/2 and GSK3β phosphorylation in vivo. These results may provide new insights into the cross-regulation of apoptosis and autophagy following radiotherapy and may aid in the design of new therapies for the treatment of NSCLC.

Materials and methods

Ethics and statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by our Institutional Animal Care and Use Committee of the Tianjin Medical University Cancer Institute & Hospital, National Clinical Research Center for Cancer, Tianjin, China (No.: 20110519).

Cell culture

The normal lung bronchial epithelial BEAS-2B cell and the NSCLC cell lines including A549, H460, H1299, H2292, and H520 were obtained from the American Type Culture Collection. All cell lines were maintained in high glucose DMEM (Invitrogen) containing 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a 5% CO2 incubator.

Plasmid construction, amplification and preparation

The plasmid encoding a short hairpin RNA (pshRNA) targeting the PKM2 was generated using the specific sequence, as reported previously [26,27]. Two sequences of shRNA targeting the PKM2 (pshRNA-PKM2) and control pshRNA (pshRNA-Con) were 5′-CCGGGCTGTGGCTCTAGACACTAAACTCGAGTTAGTGTCAGAGCTCT-GCTTTTG-3′, and 5′-CCGGGAGGCTTCTTATAAGTGTTTACTCGAGAAACACTAT-AGAGCTCT-GCTTTTG-3′, and 5′-CCGGGAGGCTTCTTATAAGTGTTTACTCGAGAAACACTAT-AGAGCTCT-GCTTTTG-3′, respectively. These sequences were cloned into the plasmid of pGenesil-2 (Genesil Biotechnology) to generate plasmids of pshRNA-PKM2 and pshRNA-Con, respectively. Following transformation, these two plasmids were extracted from E. coli and their authenticity was demonstrated by DNA sequencing. The extracted plasmids were suspended in sterile endotoxin-free water and stored at −20 °C.

Immunofluorescence assay

Briefly, A549 cells at 1 × 10⁶/ml were cultured overnight to reach 50–70% confluence on coverslips in 6-well plates. The cells were transfected with, or without, 24 μg pshRNA-PKM2 or pshRNA-Con using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Cells were irradiated at a dose of 4 Gy 48 h later. The treated cells were further cultured in complete medium for 12 or 24 h, then fixed in 4% paraformaldehyde, and blocked with 1% BSA for 30 min. The cells were permeabilized in 1% BSA-supplemented PBS containing 0.5% Triton-X100 for 20 min, washed, and incubated overnight at 4 °C with or without the specific antibodies against antibodies against p53 (1:1000, Cell Signaling Technology). The bound antibodies were detected with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized using Immobilon Western Chemiluminescent HRP Substrate (millipore). The relative levels of target protein to control β-actin were analyzed by Quantity One 1D image analysis software 4.4.0 (Bio-Rad). Additional experiments were performed in the cells that had been pre-treated 1 h prior to IR with Z-VAD or/and 3-MA as described above, respectively.

Western blot analysis

The apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using an In Situ Cell Death Detection Kit, Fluorescein (Roche) following the manufacturer’s instructions. The cells were irradiated at a dose of 4 Gy IR and the cells were transfected in triplicate with or without the plasmid for 48 h and were treated with, or without, 1 mM 3-MA for 1 h, followed by IR at a dose of 4 Gy. Some groups of cells were treated in triplicate with 4 Gy IR. One day later, the apoptotic cells were determined and five fields selected randomly were analyzed for the percentages of apoptotic cells.

Fluorescence-activated cell sorting (FACS)

The Annexin V-FITC and propidium iodide (PI) binding assay was performed to determine the apoptosis and necrosis of cells in vitro. The A549 and H460 cells were seeded in a 6-well plate at a seeding density of 2 × 10⁵ cells/ml. When the cells were adhered, different treatments were simultaneously added. After a 24-hour incubation, the cells were trypsinized, washed in PBS, and resuspended in binding buffer. The cells were incubated in the dark for 10 minutes with Annexin V-FITC (100 ng/ml) and 10 μl PI added to each group. Positive Annexin V staining indicated apoptosis, while positive PI indicated necrosis. For each group, a minimum of 30,000 cells were counted then examined using flow cytometer (FCM, BD FACS Array, San Jose, USA).

Electronic microphotography

The different concentrations of A549 cells transfected with or without plasmid as well as with or without pre-treatment with 30 μM benzoxoxy carbonylvalyl-valyl-asparyl fluoromethyl ketone (Z-VAD, R&D Systems) or 1 mM 3-methyladenine (3-MA) (Sigma) were treated in IR at a dose of 0, 2, 4, 6, and 8 Gy. Two weeks later, cells were fixed with methanol and stained with 0.1% crystal violet. The number of colonies, defined as ≥50 cells/colony, was counted. The experiments were performed in triplicate.

IR and cell viability assay

A549 and H460 cells were transfected with, or without, plasmid for 48 h, as described above, and some groups of cells were treated in triplicate with IR at a dose of 4 Gy in a 160 kV X-rays using a RS-2000 Biological Irradiation Device (Rad Source Technologies, USA) at a doserate of 8.3 Gy/min. The cells were further cultured in incomplete medium for 12 or 24 h, then fixed in 4% paraformaldehyde, and blocked with 1% BSA for 30 min. The cells were permeabilized in 1% BSA-supplemented PBS containing 0.5% Triton-X100 for 20 min, washed, and incubated overnight at 4 °C with a specific antibody against phospho-ERK(#4377S), p-GSK3β(#9323S), p-PDK1(#3438S), LC3(#4108), or β-actin (Cell Signaling Technology). The relative levels of target protein to control β-actin were analyzed by Quantity One 1D image analysis software 4.4.0 (Bio-Rad).

Immunohistochemical assay

The expression of PKM2 in tumor tissues was determined by immunohistochemical analysis. Briefly, the tumor tissues from individual groups of mice were dissected out, and tissue sections at 4 μm were rehydrated, treated with 3% H2O2 blocking with 5% non-fat milk in Tris-buffered saline (TBS), 0.1% Tween 20 for 1 h, then the membranes were incubated overnight at 4 °C with or without the specific antibodies against antibodies against PKM2 (#4053, Akt(#9272), phospho-Akt (#4695), phospho-ERK (#4377S), p-GSK3β(#9323S), p-PDK1(#3438S), LC3(#4108), or β-actin (Cell Signaling Technology). The bound antibodies were detected with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized using Immobilon Western Chemiluminescent HRP Substrate (millipore). The relative levels of target protein to control β-actin were analyzed by Quantity One 1D image analysis software 4.4.0 (Bio-Rad). Additional experiments were performed in the tumors that had been pre-treated 1 h prior to IR with Z-VAD or/and 3-MA as described above, respectively.
and blocked with 3% BSA. Subsequently, the tissue sections were incubated with anti-PKM2 antibody (1:1000, Cell Signaling Tech) or with control IgG overnight at 4 °C. The bound antibodies were detected with HRP-conjugated second antibodies and DAB, followed by imaging under a light microscope.

Statistical analyses

Data are expressed as mean ± SD. Data were analyzed using Student’s t-test. *p < 0.05 was considered significant.

Results

Knockdown of PKM2 expression increases the sensitivity of NSCLC cells to radiotherapy in vitro

To examine PKM2 expressions levels in the normal lung epithelial cell and the NSCLC cell lines, we evaluated the expression levels of PKM2 in normal lung bronchial epithelial cell BEAS-2B and five NSCLC cell lines including A549, H460, H1299, H292, and H520 by Western blotting assays, and our results demonstrated that PKM2 expression was elevated in almost five NSCLC cell lines examined compared to autologous normal lung bronchial epithelial cell, although the expression levels fluctuated slightly depending on the different cell lines (Fig. 1A).

To test the role of PKM2 in the sensitivity of NSCLC to radiotherapy, we generated plasmids of pshRNA-PKM2 and control pshRNA-Con by inserting the DNA fragment for a pshRNA specifically targeting the PKM2 or control into the pGenesil2 vector. After demonstrating the authenticity, A549 and H460 cells were transfected with the plasmid for 48 h and the levels of PKM2 expression were tested by Western blot assays. Obviously, transfection with control plasmid did not significantly modulate PKM2 expression; while transfection with pshRNA-PKM2 increased the percentages of γ-H2AX-positive cells when compared with mock-treated and control cells. These results suggested that pshRNA-PKM2 cells were more sensitive to IR than mock-treated and control cells (Fig. 1B and F).

Given that IR usually causes DNA double-strand breaks [28], we characterized the frequency of γ-H2AX nuclear foci positive cells by immunofluorescent assays. While IR treatment dramatically increased the frequency of γ-H2AX-positive cells, the same dose of IR further significantly increased the percentages of γ-H2AX-positive cells when combined with PKM2 silencing at 12 and 24 h after IR, and there was a significant difference in γ-H2AX-positive cells between these two groups at 12, 24 h after IR (Fig. 1G and H, p < 0.05). Taken together, these data clearly indicated that PKM2 silencing increased the sensitivity, unirradiated control, mock-treated, and pshRNA-PKM2-transfected A549 cells were subjected to IR (0, 2, 4, 6, and 8 Gy), and two weeks after IR, these cells were tested for the capacity for colony formation. The results showed that the numbers of colonies formed by pshRNA-PKM2 cells were significantly decreased compared with that of mock-treated and control cells; however, there was no significant change in mock-treated cells compared with control cells. These results suggested that pshRNA-PKM2 cells were more sensitive to IR than mock-treated and control cells (Fig. 1E and F).

Mock-treated and pshRNA-PKM2-transfected A549 and H460 cells were subjected to IR (4 Gy), and 12 and 24 h after IR, these cells, together with un-irradiated mock-treated, pshRNA-Con-transfected, and pshRNA-PKM2-transfected cells, were tested for cell viability by trypan blue staining. Knockdown of PKM2 reduced the percentage of A549 viable cells by 12.6–20% and IR treatment decreased the frequency of viable cells by 17.1–28.2%. However, the percentages of viable cells in the PKM2-silencing and irradiated cells were reduced by 27.7–48.7%, which were significantly lower than that in other groups (Fig. 1D, p < 0.05). Furthermore, it was consistent with the above results of A549 cells that knockdown of PKM2 significantly reduced the percentage of H460 viable cells (Appendix: Supplementary Fig.S1B).

In addition, to further validate PKM2 silencing on their radiosensitivity, unirradiated control, mock-treated, and pshRNA-PKM2-transfected A549 cells were subjected to IR (0, 2, 4, 6, and 8 Gy), and two weeks after IR, these cells were tested for cell viability together with un-irradiated mock-treated, pshRNA-Con-transfected, and pshRNA-PKM2-transfected cells, were tested for cell viability by trypan blue staining. Knockdown of PKM2 reduced the percentage of A549 viable cells by 12.6–20% and IR treatment decreased the frequency of viable cells by 17.1–28.2%. However, the percentages of viable cells in the PKM2-silencing and irradiated cells were reduced by 27.7–48.7%, which were significantly lower than that in other groups (Fig. 1D, p < 0.05). Furthermore, it was consistent with the above results of A549 cells that knockdown of PKM2 significantly reduced the percentage of H460 viable cells (Appendix: Supplementary Fig.S1B).

Mock-treated and pshRNA-PKM2-transfected A549 and H460 cells were subjected to IR (4 Gy), and 12 and 24 h after IR, these cells, together with un-irradiated mock-treated, pshRNA-Con-transfected, and pshRNA-PKM2-transfected cells, were tested for cell viability by trypan blue staining. Knockdown of PKM2 reduced the percentage of A549 viable cells by 12.6–20% and IR treatment decreased the frequency of viable cells by 17.1–28.2%. However, the percentages of viable cells in the PKM2-silencing and irradiated cells were reduced by 27.7–48.7%, which were significantly lower than that in other groups (Fig. 1D, p < 0.05). Furthermore, it was consistent with the above results of A549 cells that knockdown of PKM2 significantly reduced the percentage of H460 viable cells (Appendix: Supplementary Fig.S1B).

In addition, to further validate PKM2 silencing on their radiosensitivity, unirradiated control, mock-treated, and pshRNA-PKM2-transfected A549 cells were subjected to IR (0, 2, 4, 6, and 8 Gy), and two weeks after IR, these cells were tested for the capacity for colony formation. The results showed that the numbers of colonies formed by pshRNA-PKM2 cells were significantly decreased compared with that of mock-treated and control cells; however, there was no significant change in mock-treated cells compared with control cells. These results suggested that pshRNA-PKM2 cells were more sensitive to IR than mock-treated and control cells (Fig. 1E and F).

Given that IR usually causes DNA double-strand breaks [28], we characterized the frequency of γ-H2AX nuclear foci positive cells by immunofluorescent assays. While IR treatment dramatically increased the frequency of γ-H2AX-positive cells, the same dose of IR further significantly increased the percentages of γ-H2AX-positive cells when combined with PKM2 silencing at 12 and 24 h after IR, and there was a significant difference in γ-H2AX-positive cells between these two groups at 12, 24 h after IR (Fig. 1G and H, p < 0.05). Taken together, these data clearly indicated that PKM2 silencing increased the

![Fig. 1](image-url)

Fig. 1. The PKM2 expression levels in the normal lung epithelial cell and the NSCLC cell lines and knockdown of PKM2 expression enhance the radiosensitivity of A549 cells in vitro. The expression levels of PKM2 in normal lung bronchial epithelial cell BEAS-2B and five NSCLC cell lines including A549, H460, H1299, H292, and H520 were determined by Western blotting assay (A). A549 cells were transfected with pshRNA-PKM2 or pshRNA-Con plasmid for 48 h, and the levels of PKM2 expression were determined by Western blot assays using a PKM2-specific antibody and β-actin as an internal control (B and C). Quantitative analysis revealed that transfection with pshRNA-PKM2 significantly reduced PKM2 expressions as compared with that in the mock-treated and control pshRNA-Con plasmid-transfected cells, respectively (p < 0.05, Fig. 1C).

Mock-treated and pshRNA-PKM2-transfected A549 and H460 cells were subjected to IR (4 Gy), and 12 and 24 h after IR, these cells, together with un-irradiated mock-treated, pshRNA-Con-transfected, and pshRNA-PKM2-transfected cells, were tested for cell viability by trypan blue staining. Knockdown of PKM2 reduced the percentage of A549 viable cells by 12.6–20% and IR treatment decreased the frequency of viable cells by 17.1–28.2%. However, the percentages of viable cells in the PKM2-silencing and irradiated cells were reduced by 27.7–48.7%, which were significantly lower than that in other groups (Fig. 1D, p < 0.05). Furthermore, it was consistent with the above results of A549 cells that knockdown of PKM2 significantly reduced the percentage of H460 viable cells (Appendix: Supplementary Fig.S1B).
sensitivity of the NSCLC cells to radiotherapy in vitro. It was noted that pshRNA-Con had almost no effect on A549 cells, therefore, some subsequently experiments did not set this group.

**Knockdown of PKM2 enhances IR-induced apoptosis in NSCLC cells**

Next, we tested the impact of PKM2-silencing on IR-induced cell death types. One day after IR, the apoptotic cells in the irradiated mock-treated, pshRNA-PKM2-transfected, and one group of cells that had been pre-treated with 30 μM Z-VAD for 1 h prior to IR, together with mock-treated, unirradiated pshRNA-Con-transfected, and pshRNA-PKM2-transfected groups of cells were characterized by TUNEL assays and/or FACS analysis (Fig. 2A and C). In comparison with that in mock-treated and control plasmid-transfected cells, the frequency of apoptotic cells in the PKM2 silencing or IR-treated cells increased moderately, while the percentages of apoptotic cells in the cells receiving combined treatment with IR and PKM2-silencing were significantly greater. However, the frequency of apoptotic cells in the Z-VAD-pretreated cells was partially reduced. Apparently, knockdown of PKM2 and IR induced apoptosis in NSCLC cells in vitro (Fig. 2B and D, and Appendix: Supplementary Fig. S1C).

**Knockdown of PKM2 enhances IR-induced autophagy in NSCLC cells**

The cell autophagy is characterized by the formation of numerous autophagic vacuoles, autophagosome, in the cytoplasm and elevated levels of the microtubule-associated protein 1 light chain 3 (LC3)-II [29]. To test the impact of PKM2 silencing on IR-induced autophagy, the presence of autophagosome in mock-treated, pshRNA-Con-transfected, pshRNA-PKM2-transfected, IR-treated alone, IR + pshRNA-PKM2-transfected, and 1 mM 3-MA-pretreated IR + pshRNA-PKM2-transfected cells was characterized by electronic microphotography (EM). Intriguingly and importantly, numerous autophagosomes were detected in the IR + pshRNA-PKM2-transfected cells, and only a few were detected in the untreated control group.
pshRNA-PKM2-transfected, IR-treated alone, and 3-MA-pretreated cells, but not in the other groups of cells (Fig. 3A). Western blot analyses indicated that the relative ratios of LC3-II to LC3-I increased in the irradiated and PKM2-silencing cells, particularly in the IR + pshRNA-PKM2-transfected cells (Fig. 3B and Appendix: Supplementary Fig. S1D). However, pre-treatment with 3-MA dramatically decreased the ratio of LC3-II to LC3-I. These data clearly demonstrated that knockdown of PKM2 and IR significantly induced autophagy in NSCLC cells in vitro.

To understand the mechanisms underlying the action of PKM2 silencing in the process of autophagy [30–36], we characterized the activation of the PI3K/Akt and Raf-1/MEK1/2/ERK1/2 pathways by Western blot assays. There was no obvious difference in the levels of AKT and ERK1/2 expression in the different groups of cells. Either PKM2 silencing or IR slightly reduced the levels of AKT phosphorylation, but did not obviously affect the levels of ERK1/2, GSK3β, and PDK1 phosphorylation. In contrast, the combination of PKM2 silencing with IR dramatically reduced the levels of AKT and PDK1 phosphorylation, but obviously elevated the levels of ERK1/2 and GSK3β phosphorylation. Apparently, the inhibition of AKT and PDK1 activation, together with slightly increased ERK and GSK3β activation, contributed to the PKM2-silencing and IR-induced autophagy in A549 cells in vitro (Fig. 3C).

Cross regulation of PKM2-silencing and IR-induced apoptosis and autophagy in NSCLC cells

To investigate the potential cross regulation between the PKM2-silencing and IR-induced autophagy and apoptosis, A549 and H460 cells were transfected with pshRNA-Con or pshRNA-PKM2 for 48 h and pre-treated with 1 mM 3-MA or/and 30 μM Z-VAD 1 h, followed by IR. The cell viability, the capacity for colony formation, and the frequency of apoptotic and autophagic cells in the different groups of cells were detected. Pre-treatment with 3-MA or Z-VAD only slightly reduced the percentages of viable control cells, but significantly increased the frequency of viable cells, as compared with that in the irradiated PKM2-silencing cells (Fig. 4A). In addition, pre-treatment with 3-MA or Z-VAD significantly reduced the capacity for colony formation compared with mock-treated cells, but significantly increased the capacity for colony formation, as compared with that in the irradiated PKM2-silencing cells (Fig. 4A). Among the combination groups, pre-treatment with 3-MA and Z-VAD led to the significant increase in the frequency of viable cells and the capacity for colony formation compared to pre-treatment with 3-MA or Z-VAD (Fig. 4A and B).

Similarly, pre-treatment with 3-MA only moderately increased the percentage of apoptotic control cells, while the same
treatment significantly reduced the frequency of apoptotic PKM2-silencing irradiated cells (Fig. 4C). In parallel, pre-treatment with Z-VAD did not modulate the relative levels of LC3-II and LC3-I in control cells, and the same treatment dramatically reduced the relative ratios of LC3-II to LC3-I in irradiated PKM2-silencing cells (Fig. 4D). These data clearly indicated that there was a cross regulation between the PKM2-silencing and IR-induced apoptosis and autophagy in NSCLC cells in vitro.

Knockdown of PKM2 expression and IR inhibit the growth of implanted tumors by inducing apoptosis and autophagy in mice

To determine the effect of PKM2 silencing on the radiosensitivity in vivo, nude mice were inoculated subcutaneously with $1 \times 10^6$ A549 cells, and when the implanted tumors grew at 50 mm$^3$ in one dimension, the mice were randomized and treated intravenously with vehicle as Controls, pshRNA-Control, pshRNA-PKM2, IR alone or in combination with pshRNA-Control, and irradiation (pshRNA-PKM2 + IR), respectively. The body weights and the growth of implanted tumors in individual mice were monitored longitudinally, and the body weights in different groups of mice were indistinguishable at individual time points (Fig. 5A). Treatment with control plasmid did not affect the growth of implanted tumors, but IR or treatment with pshRNA-PKM2 significantly inhibited the growth of implanted tumors in mice. More interestingly, treatment with pshRNA-PKM2 and IR significantly suppressed the growth of implanted tumors in mice (Fig. 5B and C).

Furthermore, characterization of apoptotic cells in the dissected tumor indicated that, while either IR or treatment with pshRNA-PKM2 induced a moderate frequency of tumor cell apoptosis, combination of them significantly increased the frequency of apoptotic cells (Fig. 5D). Similarly, obvious more autophagosomes were observed in the tumors that had been treated with pshRNA-PKM2 and IR than in single treatment (Fig. 5E). Finally, analysis of PKM2 expression revealed that the levels of PKM2 in the pshRNA-PKM2-treated tumors were significantly reduced (Fig. 5F and G). Together, these data indicated that treatment with pshRNA-PKM2 silenced PKM2 expression, and PKM2-silencing and IR significantly induced tumor cell apoptosis and autophagy, leading to the inhibition of implanted tumors in mice.

Discussion

Combination of different treatment strategies for the treatment of cancer has advantages. It has long been well established that inhibition of glycolysis via 2-deoxy-D-glucose (2-DG), an inhibitor of glucose transport and glycolysis, improves the efficacy of radiotherapy without obvious damage to normal tissues [7–23]. Recent studies have shown that PKM2 is exclusively expressed in cancer-tissue specific and is necessary for aerobic glycolysis [6,21].
PKM2 is important for cancer metabolism and tumor growth, and contributes to the development of radioresistance by generating a chemically reduced milieu \[6,21\]. Indeed, isotype-specific inhibition of the transition between the tetrameric and dimeric forms of PKM2 inhibits cancer cell proliferation \[24,25\]. Furthermore, nuclear translocation of the PKM2 induces a caspase- and Bcl-2-family member-independent apoptosis of cancer cells \[37\]. In addition, inhibition of PKM2 activity enhances chemotherapeutic drug-inhibited growth of tumors in vivo \[26,27\]. In this study, we generated a plasmid for specifically targeting PKM2 expression and examined the impact of PKM2 silencing on radiosensitivity of NSCLC cells and the potential mechanisms underlying the action of PKM2 in regulating IR-induced autophagy and apoptosis in vitro and in vivo. We found that knockdown of PKM2 expression enhanced IR-induced apoptosis and autophagy in vitro and in vivo, accompanied by inhibiting AKT and PDK1 phosphorylation, but enhancing ERK and GSK3β phosphorylation in NSCLC cells.

Autophagy, a process whereby cells maintain homeostasis by eliminating unnecessary proteins and damaged organelles, can be triggered by physiological factors, such as nutrient starvation, or by a variety of stimuli, such as IR and cytotoxic drugs \[34,35,38,39\]. Autophagy is characterized by the appearance of autophagosomes that contain the cytoplasmic materials and organelles, commonly observed by electron microscopy, the gold standard method \[40\]. In addition, LC3 is a known marker of autophagosomes \[41\]. Previous studies have demonstrated that induction of autophagy is basically a protective mechanism to support cell survival under adverse environmental conditions and is associated with the development of radioresistance \[42,43\]. However, the impact of autophagy on the sensitivity of tumor cells to IR remains controversial. While blockage of autophagy improves the sensitivity of carcinoma cells to IR \[42,43\], recent studies have indicated that induction or enhancement of autophagy increases radiosensitivity in several types of cancer cells \[44,45\]. Furthermore, several antitumor drugs have been shown to induce autophagy and improve radiosensitivity, independent of apoptosis \[30–32,44–50\]. In the present study, we showed that combination of IR with PKM2 silencing had higher cytotoxicity against NSCLC line by enhancing IR-induced autophagy and apoptosis in NSCLC cells. Evidently, higher ratios of LC3-II to LC3-I expression and higher frequency of cell apoptosis were detected in IR-treated PKM2-silencing NSCLC cells. Our

**Fig. 5.** Treatment with pshRNA-PKM2 enhances the IR-inhibited growth of implanted tumors in mice. The nude mice were inoculated with A549 cells and when the tumor grew at 50 mm³ in one dimension, the mice were randomized and treated with vehicle (PS), plasmid of pshRNA-Con or pshRNA-PKM2 alone or IR (4 Gy × 7) alone or in combination with pshRNA-PKM2 and IR, respectively. The body weights and tumor growths of individual mice were monitored longitudinally. At the end of the in vivo experiment, the tumor tissues were dissected out and the frequency of apoptotic cells, the presence of autophagosomes and the expression of PKM2 were determined by TUNEL, EM and immunohistochemistry, respectively. Data are representative images or expressed as mean ± SD of individual groups of mice (n = 6 per group). (A) The body weights of mice. (B and C) The tumor growth curve of implanted tumors and the log-transformed tumor growth curve of implanted tumors in mice. (D) Quantitative analysis of the frequency of apoptotic cells. (E) EM analysis of autophagy. (F) The expression of PKM2. (G) Quantitative analysis of PKM2 expression. The cells with brown cytoplasm were considered as positive anti-PKM2 staining and the percentage of PKM2-positive cells was obtained by dividing the numbers of the PKM2-positive cells by the total number of cancer cells in the same field.
data were consistent with the observations that enhanced autophagy improves the radiosensitivity of tumor cells.

Currently, several signaling pathways are involved in the regulation of autophagy. Tritrepodin B-group soyasaponins, curcumin, and MG-2477 (a new tubulin inhibitor) can induce autophagy in different types of cancer cells by inhibiting the AKT/mTOR signaling, but enhancing the ERK1/2 activity [33,51,52]. Similarly, inhibition of the PI3K/mTOR signaling by NVP-BGT226 can induce autophagy [53]. We found that treatment with both PKM2-silencing plasmid and IR obviously inhibited the AKT and PDK1 signaling, but increased the ERK1/2 and GSK3β phosphorylation in A549 cells. Apparently, enhanced ERK signaling and down-regulated AKT signaling are associated with induction of autophagy [54].

The relationship between apoptosis and autophagy is complex and varies among different types of cells, according to the stimuli the cells face [34,35,39,40,55]. A stimulus can induce either apoptosis or autophagy alone or both in the same cells concurrently or sequentially [30–32,39,50,55]. Induction of autophagy can progress into apoptosis [50,55–59]. A recent study has indicated that autophagy protects breast cancer cells from epirubicin-induced apoptosis and serves as a survival pathway in a Myc-induced model of lymphoma, following treatment with apoptosis activators [36]. However, recent studies have shown that IR combined with arsenic trioxide enhances cell-killling effects in human cancer cells through induction of both autophagy and apoptosis [30–32]. Indeed, we found that treatment with 3-MA reduced the frequency of IR/phsrNA-PKM2-induced apoptotic NSCLC cells. Similarly, pre-treatment with Z-VAD also reduced the cytotoxicity of IR combined with phsrNA-PKM2, but elevated the relative levels of LC3-II to LC3-I in IR-treated PKM2-silencing cells. The greater ratios of LC3-II to LC3-I indicated that inhibition of apoptosis enhanced drug-induced autophagy in IR-treated PKM2-silencing cells. We found that pre-treatment with either 3-MA or Z-VAD increased the survival of IR-treated PKM2-silencing NSCLC cells. Treatment with 3-MA induced NSCLC cell apoptosis, but mitigated IR-induced apoptosis in PKM2-silencing cells. On the other hand, treatment with Z-VAD failed to modulate spontaneous autophagy, but inhibited IR-induced autophagy in PKM2-silencing cells. Apparently, inhibition of apoptosis also minimized IR-induced autophagy, while inhibition of autophagy abrogated IR-induced apoptosis in cells in our experimental conditions. Our data suggest that there is a positive cross-regulation between IR-induced apoptosis and autophagy in PKM2-silencing NSCLC cells. Given that IR can induce both apoptosis and autophagy, IR combined with either an apoptosis or autophagy inducer may enhance the sensitivity of tumor cells to radiotherapy.

More importantly, we found that treatment with plasmid for silencing PKM2 or IR inhibited the growth of implanted tumors in mice and that treatment with both PKM2-silencing plasmid and IR significantly reduced the volumes of implanted tumors in mice. Furthermore, we found that treatment with PKM2-silencing plasmid dramatically reduced the levels of PKM2 expression in tumor tissues and that treatment with both the plasmid and IR induced significantly higher frequency of tumor cell apoptosis and promoted obviously more autophagosomes in tumor cells. Our data are consistent with previous findings that treatment with 2-DG enhances IR-related cytotoxicity and improves radiotherapy for cancers [19,20,60–64]. These data extend our previous findings that combination of PKM2 inhibition with chemotherapy inhibits the growth of implanted tumors in vivo [26,27]. Therefore, IR combined with PKM2 inhibition may be a promising strategy potential for enhancing radiotherapy of NSCLC and other types of cancers.

In summary, our results indicated that treatment with both PKM2 silencing and IR enhanced the radiotherapeutic efficacy in NSCLC cells by inducing autophagy and apoptosis, which was associated with inhibition of the AKT signaling, and enhancement of the ERK signaling. Pre-treatment with either Z-VAD or (and) 3-MA reduced IR-related cytotoxicity, autophagy and apoptosis in PKM2-silencing cells, suggesting a positive cross-regulation between IR-related autophagy and apoptosis processes. More importantly, treatment with both PKM2 silencing and IR significantly inhibited the growth of implanted tumors in mice by promoting autophagy and apoptosis. Although 2-DG and the lactate dehydrogenase inhibitor oxamate might be used to inhibit glycolysis and obtain a significant therapeutic gain in combination treatments with cytotoxic drugs or radiotherapy; the present study had potential weaknesses such as transient transfection may impact the interference effects of PKM2 in vivo, our findings extended previous findings and supported the notion that treatment with glycolysis inhibitor can enhance the radiosensitivity of tumor cells. These findings may provide new insights into the cross-regulation of autophagy and apoptosis following radiotherapy of cancers and may aid in the design of new therapies for the treatment of NSCLC.

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Conflict of interest

None.

Approval/disclosures

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.11.016.

References


