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ORIGINAL RESEARCH

Targeted Inhibition of HK-II Reversed the Warburg Effect to Improve the Radiosensitivity of Laryngeal Carcinoma

Xiao-Hong Chen[1]
Ding-Li Yu[2]
Jiang-Tao Zhong[2]
Shui-Hong Zhou[2]
Jun Fan[3]
Zhong-Jie Lu[4]

[1]Department of Otolaryngology, The Second Hospital of Jiaxing (The Second Affiliated Hospital, Jiaxing University), Jiaxing City, Zhejiang Province, 314000, People's Republic of China; [2]Department of Otolaryngology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang, 310003, People's Republic of China; [3]State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang, 310003, People's Republic of China; [4]Department of Radiotherapy, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang, 310003, People's Republic of China

Purpose: Hexokinase-II (HK-II) is the key enzyme in the first rate-limiting step of glycolysis that catalyzes the conversion of glucose to glucose-6-phosphate. Here, we examined the association between HK-II expression and radioresistance in laryngeal carcinoma and whether the inhibition of HK-II expression can enhance the radiosensitivity of these tumors.

Methods: The effects of HK-II small interfering RNA (siRNA) on the radiosensitivity of Tu212 cells were examined in vitro and in vivo in a mouse model. Cells were irradiated using a 6-MV linear accelerator. The cell viability, cell survival, proliferation, apoptosis, and cell cycle of Tu212 cells were evaluated using trypan blue staining, colony formation assays, CCK-8 assays, and flow cytometry, respectively. Oxygen consumption, lactic acid production, glucose consumption, and the ATP level of Tu212 cells were also examined. The expression of glycolytic and regulatory enzymes involved in the tricarboxylic acid cycle was assessed using Western blotting.

Results: The HK-II siRNA and X-ray combination treatment led to a significantly greater reduction of cell viability, inhibition of cell survival and proliferation, increased apoptosis, and increased G2 phase arrest compared to either treatment alone (all, $P<0.01$). HK-II siRNA increased the oxygen consumption rate of cells, significantly inhibited lactic acid production and glucose consumption, and significantly suppressed the upregulation of HK-II, pyruvate kinase M2 (PKM2), pyruvate dehydrogenase (PDH), phosphofructokinase platelet (PFKP), lactate dehydrogenase (LD), and citrate synthase (CS) (all, $P<0.01$).

Conclusion: The inhibition of HK-II by siRNA enhances the radiosensitivity of laryngeal carcinoma Tu212 cells by inhibiting glycolysis and partially inhibiting oxidative phosphorylation.

Keywords: laryngeal carcinoma, hexokinase-II, siRNA, radiosensitivity, Warburg effect

Introduction

Radiotherapy plays a major role in the treatment of laryngeal carcinoma, and radioresistance leads to treatment failure in some patients.[1–5] However, the mechanism underlying radioresistance in laryngeal carcinoma remains unclear. The Warburg effect, ie, glycolysis in cancer cells, increases even under aerobic conditions and is involved in radioresistance in some cancers.[6–10] The Warburg effect has been shown to be related to radioresistance in the head and neck squamous cell carcinoma cell line, ie, RSCC6-1 cells.[10]

The most obvious characteristic of the Warburg effect is aerobic glycolysis.[6–10] Although the mechanism underlying elevated glycolysis in cancer cells remains

Correspondence: Shui-Hong Zhou
Department of Otolaryngology, The First Affiliated Hospital, College of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou, 310003, People's Republic of China
Tel +86-571-87236894
Fax +86-571-87236895
Email 1190051@zju.edu.cn

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unclear, the most likely mechanism involves increased activities of rate-limiting enzymes in the intracellular glycolysis pathway of malignant tumors after glucose uptake, especially hexokinase (HK).[6] HK is a focus of attempts to enhance the radiosensitivity of tumor cells.[6]

HK is a key limiting enzyme of glycolysis and exists as four isozymes: HK-I, HK-II, HK-III, and HK-IV. HK-II has been shown to be closely related to malignant tumors. HK-II is the key enzyme in the first rate-limiting step of glycolysis that catalyzes the conversion of glucose to glucose-6-phosphate.[6] HK-II is highly expressed in many malignant tumors, and its high level of expression was shown to be related to cell proliferation, invasion, metastasis, recurrence, and poor prognosis.[11–13] Some studies have shown that high levels of HK-II expression are also associated with chemoradioresistance.[6,14–16] In laryngeal carcinoma, high levels of HK-II expression are associated with the TNM stage, and the inhibition of HK-II expression may suppress the growth of laryngeal carcinoma.[17–20] Min et al reported that inositol polyphosphate 4-phosphatase (INPP4B) mediates radioresistance in laryngeal carcinoma through increasing glycolysis induced by HK-II.[21] However, the inhibition of HK-II alone did not improve the chemoradiosensitivity of laryngeal carcinoma, whereas co-depletion of INPP4B and HK-II markedly enhanced the chemoradiosensitivity of laryngeal carcinoma.[21] Thus, the role HK-II plays in radioresistance in laryngeal carcinoma needs to be investigated further.

In the present study, we investigated whether HK-II expression is associated with radioresistance in laryngeal carcinoma and whether siRNA-mediated inhibition of HK-II expression can enhance the radiosensitivity of these tumors.

Materials and Methods

This study was conducted in accordance with the guidelines of, and approved by the Second Hospital of Jiaxing City, Jiaxing City, Zhejiang Province, China (approval number: jxey-2017013).

Patient Samples

Twelve pathological specimens from patients with laryngeal squamous cell carcinoma (LSCC) and 12 samples of paracarcinoma tissue (taken 0.5 cm from the negative margin after tumor excision) treated at our hospital between September 2010 and November 2016. All participants provided informed consent to be included in this study, in accordance with the Declaration of Helsinki.

Immunohistochemical Method

Paraffin sections were cut at 5 μm thickness. After deparaffinization and hydration, the sections underwent antigen retrieval by the microwave-oven method. Endogenous peroxidase activity was blocked 20 min at room temperature. Primary antibodies against HK-II (1:200) overnight. The sections were stained using a DAB Staining Kit and subjected to hematoxylin-and-eosin staining. The sections were photographed under a microscope; cells labeled with yellow or brownish-yellow granules were considered positive. Five high-magnification fields (×400) were randomly selected, in each of which 100 cells were counted; scoring was as follows: 0<10%; 1, 11%~50%; and 2, >50% positive cells. Dye depth was scored as follows: 0, no staining; 1, light yellow; 2, brownish-yellow; and 3, deep brownish-yellow. Immunohistochemical data were assessed as the positive-cell score + the dye-depth score. Total scores of 0–3 were considered negative (–), 4 was positive (+), respectively. The investigator was blinded to the group allocation.

Cell Culture

Human laryngeal squamous cell carcinoma Tu212 cells were purchased from the Cell Research Institute of the Chinese Academy of Sciences (Shanghai, China). Tu212 cells were cultured in culture medium containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 100 g/mL streptomycin, and 100 U/mL penicillin at 37°C in an atmosphere containing 5% CO_2. The cells were trypsinized and harvested after reaching 80–90% confluence, and the cell concentration was adjusted to 10^7/mL.

Experimental Groups

Cultures were divided into 10 groups for this experiment: negative control (NC) siRNA, NC siRNA+2Gy, NC siRNA+4Gy, NC siRNA+8Gy, NC siRNA+16Gy, HK-II siRNA, HK-II siRNA+2Gy, HK-II siRNA+4Gy, HK-II siRNA+8Gy, and HK-II siRNA+16Gy. Cells were irradiated using a 6-MV linear accelerator (Clinac 23EX; Varian Inc., Palo Alto, CA, USA) at 24 hours posttransfection. The irradiation treatment (source dish distance: 100 cm; radiation field: 35 × 35 cm; single X-ray dose: 6 MV; dose rate: 500 mU/min; dose: 2, 4, 8, or 16 Gy) was performed in triplicate for each condition and the cells were cultured for 48 hours.[23]

Trypan Blue Staining

The cells were collected and centrifuged at 1000 rpm for 1 minute. The supernatant was discarded, and the cells

were resuspended in 1 mL of cell suspension medium. Aliquots of 100 μL of trypan blue solution were added to 100 μL of resuspended cells and allowed to mix for 3 minutes. The stained cells were counted with a hemocytometer. Cell viability was calculated as follows: (total number of cells – number of stained blue cells)/total number of cells × 100%.

Colony Formation Assay

The percentage of individual cells was adjusted to > 95% by repeating the cell suspension process. The cell suspension was diluted, and 500 cells in 2 mL of culture solution were added to six-well plates. The plates were placed in an incubator for 2–3 weeks, and the medium was replaced every 3 days. The culture was terminated when clones became visible. The cells were gently washed twice with phosphate buffered saline (PBS), stained with 1% crystal violet for 1 hour at room temperature, and photographed for counting.

Cell-Counting Kit-8 Assay

Cells in each group were incubated for 48 hours at 37°C in an atmosphere containing 5% CO_2. To the cells, 20 μL of Cell-Counting Kit-8 (CCK-8) solution was added, and the mixture was incubated for 1 hour in the dark. The absorbance at 450 nm (A_{450}) for each well was measured using a microplate analyzer, and a cell growth curve was plotted.

Flow Cytometry

Cell suspensions were collected and washed before 300 μL of binding buffer was added, followed by 5 μL of Annexin V-FITC, and the mixture was incubated in the dark at room temperature for 15 minutes. Then, 5 μL of propidium iodide and 200 μL of binding buffer were added. Apoptosis was detected in triplicate samples using flow cytometry.

Detection of Oxygen Consumption

Tu212 cells in the logarithmic growth phase were sampled and counted after undergoing digestion. The cells were cultured in a 5% CO_2 incubator for 24 hours. Adherent cells were treated in groups for 48 hours. The cells were digested every 10 minutes from 0 to 180 minutes to obtain single-cell suspensions. Oxygen consumption by the cells within 180 minutes was measured using a Seahorse XF_P analyzer (Agilent Technologies, Santa Clara, CA, USA).

Lactic Acid Detection

Aliquots of 5×10^6 cells were added to 1 mL of extract I (lactic acid array kit, BC2235; Solarbio, Beijing, China) and lysed via ultrasonication in an ice bath (power: 300 W; ultrasound time: 3 s; interval: 7 s; total time: 3 minutes). The cells were centrifuged at 12,000 rpm and 4°C for 10 minutes. A 0.8-mL aliquot of the resultant supernatant was added to 0.15 mL of extract II. The cells were centrifuged at 4°C for 10 minutes and the optical density at 570 nm (OD_{570}) of the supernatant was determined using a spectrophotometer.

Determination of Glucose Contents

The supernatant was collected and added to 1 mL of distilled water. The cells were lysed via ultrasonication in an ice bath (power: 200 W; ultrasound time: 3 s; interval: 10 s; total runs: 30 times) and placed in a heated water bath at 95°C for 10 minutes. The samples were then centrifuged at 8000 rpm and 25°C for 10 minutes, and the supernatant was examined using a glucose content assay kit (BC2505; Solarbio). The glucose content was determined by measuring the OD_{505} using spectrophotometry.

ATP Detection

The cells were digested with 0.25% trypsin, collected, and centrifuged. The collected cells were added to lysis buffer and centrifuged at 12,000 rpm at 4°C for 10 minutes, after which 100 μL of ATP solution was added to the supernatant (ATP Array Kit, S0026; Beyotime, Shanghai, China). ATP contents were determined using a luminometer with reference to a standard curve.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

siRNA Sequences

HK-II siRNA-1 (GenePharma, Shanghai, China), 5′-CTG TGAAGTTGGCCTCATT-3′; HK-II siRNA-2, 5′-ACGACA GCATCATTGTTAA-3′; HK-II siRNA-3, 5′-CTGGCTA ACTTCATGGATA-3′.

Primers

HK-II, forward 5′-CCGTGGTGGACAAGATAAG AGAGAACC-3′ and reverse 5′-GGACACGTCACATT TCGGAGCCAG-3′; GAPDH, forward 5′-ACAGTCAGC CGCATCTTCTT-3′ and reverse 5′-ACGACCAAATCC GTTGACTC-3′; voltage-dependent anion channel (VDAC)-

1, forward 5′-ACGTATGCCGATCTTGGCAAA-3′ and reverse 5′-TCAGGCCGTACTCAGTCCATC-3′.

Extraction of Total RNA

The total RNA of cells after transfection with HK-II siRNA was extracted using TRIzol reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Briefly, cells were collected and TRIzol was added. Chloroform was then added to the cells followed by centrifugation. Isopropanol was added to the supernatant, and the mixture was centrifuged at 12,000 rpm. To the resultant supernatant, 1 mL TRIzol and 1 mL 75% ethanol was then added. The RNA concentration was determined using spectrophotometry.

Reverse Transcription of mRNA

RNA was incubated in a water bath at 65°C for 5 minutes and then immediately cooled on ice. The reaction mixture consisting of 0.5 pg–1 μg of mRNA, 0.5 μL of Primer Mix, 0.5 μL of RT Enzyme Mix, and 2 μL of 5× RT buffer made up to 10 μL with nuclease-free water was incubated in a water bath at 37°C for 15 minutes, followed by incubation in a water bath at 98°C for 5 minutes, and the samples were stored at 4°C or –20°C.

Quantitative Real-Time Fluorescence Analysis

mRNA was subjected to quantitative real-time fluorescence analysis in a volume of 50 μL consisting of 5 μL of cDNA, 2 μL each of forward and reverse primers, 25 μL of SYBR® Green Realtime PCR Master Mix, and 16 μL of nuclease-free water.

Western Blotting

Cells were collected via centrifugation at 1500 rpm for 3 minutes and lysed with radioimmunoprecipitation assay lysis buffer on ice for 30 minutes followed by centrifugation at 1200 rpm and 4°C for 30 minutes. After the protein content was quantified, 4× sodium dodecyl sulfate (SDS) loading buffer was added to each sample, which was boiled for 10 minutes and centrifuged at 1200 rpm for 1 minute. The protein samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. Nonspecific labeling was blocked by placing the membranes into blocking buffer. Primary antibodies against HK-II (1:1000), pyruvate kinase M2 (PKM2, 1:1000), pyruvate dehydrogenase (PDH, 1:1000), phosphofructokinase 1 platelet isoform (PFKP, 1:1000), lactate dehydrogenase (LD, 1:1000), and citrate synthase (CS, 1:1000) were added, and the samples were incubated at 4°C overnight. GAPDH was used as an internal control. After washing three times with PBS plus Tween® 20 for 10 minutes each time, secondary antibodies were added for 2 hours at room temperature. The PVDF membranes were placed on plates for color development, and enhanced chemiluminescence substrate (liquid A and liquid B mixed in equal volumes) was dropped onto the surface of each membrane. Signals were observed using a chemiluminescence instrument.

Animals

Male athymic BALB/c nude mice (6–8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and housed in a specific pathogen-free room under conditions of controlled temperature (20°C ± 2°C) and humidity. All animal experiments were approved by the Animal Welfare Committee of our research organization (approval number: jxey-2017013). After a 1-week period of acclimatization with ad libitum access to food and water, approximately 0.2 mL of Tu212 cells (2×10^7/mL) was inoculated subcutaneously into the right flank of each mouse. The mice were divided into four groups: the Tu212+NC siRNA group, Tu212+NC siRNA+8Gy group, Tu212+HK-II siRNA group, and Tu212+HK-II siRNA+8Gy group. Each group consisted of three mice. When the tumor volume reached 100 mm^3, the mice received HK-II siRNA injections and/or X-ray treatment. Each mouse in the Tu212+HK-II siRNA group received intraperitoneal administration of 0.3 mg/kg HK-II siRNA three times per week at 2-day intervals. The X-ray irradiation groups received X-ray treatment at the same time as the first administration of siRNA and a dose of 4 Gy per week thereafter. Two weeks later, the mice were sacrificed, and the tumors were completely dissected and weighed. Tumor volume was calculated as $1/2 \times (\text{shortest diameter})^2 \times$ (longest diameter) [mm]. The tumor tissues were stored at –80°C for subsequent experiments.

TUNEL Assay

Tissue sections were fixed using a fixation solution. The sections were deparaffinized in xylene for 10 minutes and dehydrated with anhydrous ethanol for 5 minutes, 90% ethanol for 2 minutes, and 70% ethanol for 2 minutes. The sections were treated with 20 μg/mL DNase-free proteinase K at 37°C for 15 minutes and incubated with peroxidase blocking solution for 20 minutes. The sections were then incubated at

37°C for 60 minutes in the dark, after which 50 μL of TUNEL detection solution was added. Next, 100 μL of reaction-stopping solution was added, and the sections were incubated at room temperature for 10 minutes. The samples were treated with 50 μL of streptavidin-horseradish peroxidase conjugate and incubated at room temperature for 30 minutes, followed by staining with 200 μL of DAB solution before they were photographed under a fluorescence microscope.

Statistical Analysis

All in vitro experiments were performed with at least three independent replicates. Data are expressed as the mean ± standard error and were analyzed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

HK-II Expression in LSCC Tissues

HK-II expression in LSCC tissues was higher than that in paracarcinoma tissues [83.33% (10/12) VS 8.33% (1/12), p<0.05, Figure 1).

Effects of HK-II on Radiosensitivity in Tu212 Cells in vitro

Three HK-II siRNAs were designed and used to transfect Tu212 cells. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) showed that the three HK-II siRNAs inhibited the expression of HK-II, with HK-II siRNA-2 having the greatest inhibitory effect (Figure 2A). The expression of HK-II in Tu212 cells treated with HK-II siRNA-2 and/or radiotherapy was examined using Western blotting. The data showed that radiotherapy increased the HK-II protein level in Tu212 cells in a dose-dependent manner up to 8 Gy, whereas the HK-II protein level was lower following irradiation at 16 Gy compared to 8 Gy (Figure 2B). The proliferation and apoptosis of cells transfected with HK-II siRNA-2 were examined via CCK-8 assays, colony formation assays, flow cytometry, and trypan blue staining. The results of CCK-8 and colony formation assays showed that X-rays had dose-dependent inhibitory effects on the proliferation and viability of Tu212 cells. Treatment with HK-II siRNA appeared to inhibit the proliferation of Tu212 cells. The inhibitory effect of the combination of HK-II siRNA with X-ray irradiation was greater than that with either treatment alone (Figure 3A and B). Flow cytometry also showed that X-ray or HK-II siRNA treatment promoted cell cycle arrest in the G2 phase, and the effect was greater in cells treated with a combination of HK-II siRNA and X-ray irradiation than with either treatment alone (Figure 3C). The results of trypan blue staining showed that X-ray irradiation had dose-dependent cytocidal effects on Tu212 cells. Treatment with HK-II siRNA also resulted in Tu212 cell death, and the cell-killing effect of the combination of HK-II siRNA with X-rays was greater than that with either treatment alone (Figure 3D). The results of flow cytometry revealed that exposure to X-rays increased the apoptosis of Tu212 cells in a dose-dependent manner. Treatment with HK-II siRNA also increased the apoptosis of Tu212 cells, and the combination of HK-II siRNA with X-ray irradiation produced a greater effect than either treatment alone (Figure 3E).

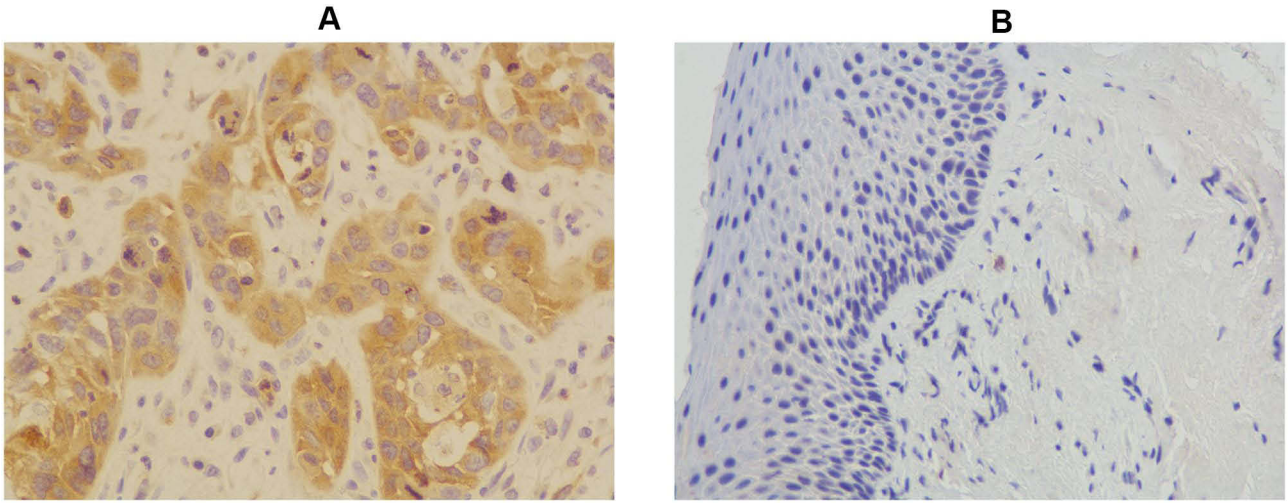

Figure 1 HK-II expression in laryngeal carcinoma tissues (**A**) and in paracarcinoma tissues (**B**).

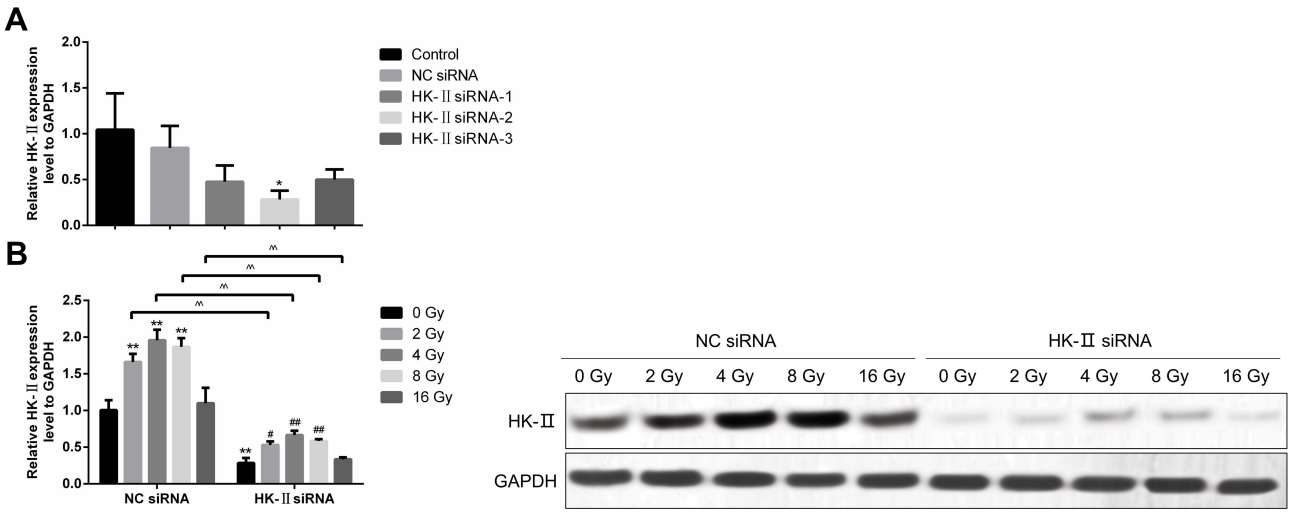

Figure 2 Verification of HK-II siRNA. (**A**) qRT-PCR results. (**B**) Western blotting results. (*p<0.05, **p<0.01, #p<0.05, ##p<0.01, and ^^p<0.01).

Thus, HK-II siRNA was shown to enhance the radiosensitivity of Tu212 cells in vitro.

4Gy irradiation has the strongest promoting effect on HK-II expression of laryngeal carcinoma TU212 cells. HK-II siRNA can improve the sensitivity of TU212 cells to 4Gy irradiation. Thus, 4Gy irradiation is used as the radiotherapy treatment dose in rescue experiment. The results of rescue experiment showed that 4Gy irradiation could significantly promote the expression of HK-II in TU212 cells. HK-II siRNA could inhibit the upregulated expression of HK-II induced by 4Gy irradiation, reduce the activity and clonogenesis ability, and enhance the apoptotic rate of Tu212 cells under radiotherapy (Figure 4). These finding demonstrated that HK-II siRNA could enhance the radiosensitivity of Tu212 cells. The results were similar as the results of Figure 2. However, HK-II overexpression plasmid could markedly suppress the down-regulated expression of HK-II in the Tu212 cells induced by HK-II siRNA. HK-II overexpression plasmid could remove the inhibitory effect on activity and clonogenesis ability of TU212 cells induce by HK-II siRNA. HK-II overexpression plasmid could also remove the promoting effect on apoptosis induced by HK-II siRNA (Figure 4). These results showed that HK-II overexpression plasmid could reduce the radiosensitivity of TU212 cells. The rescue experiment showed that the radiosensitivity of Tu212 cells was closely related to the expression of HK-II. Thus, it was suggested that reducing the expression of HK-II in laryngeal carcinoma cells can be an important approach or strategy to improve radiosensitivity of laryngeal carcinoma.

Effects of HK-II siRNA on Radiosensitivity in Tu212 Cells in vivo

Similar to the results of the in vitro experiments, we also found that X-ray treatment promoted the expression of HK-II in xenograft tumors at both the mRNA and protein levels (both $P<0.01$) (Figure 5A and B), and HK-II siRNA significantly inhibited the expression of HK-II at both the mRNA and protein levels in xenograft tumors in the presence or absence of radiotherapy (all, $P<0.01$) (Figure 5A and B). X-ray irradiation or HK-II siRNA alone significantly inhibited the growth of xenograft tumors (both, $P<0.01$) (Figure 5C and D), and the combination of HK-II siRNA with X-rays markedly inhibited tumor growth to a greater extent than either treatment alone ($P<0.01$) (Figure 5C and D). The TUNEL assay showed that X-ray irradiation or HK-II siRNA alone increased apoptosis in xenograft tumors, and the combination of HK-II siRNA with X-rays significantly increased the apoptosis level compared to either treatment alone ($P < 0.01$) (Figure 5E). Thus, HK-II siRNA enhanced the radiosensitivity of Tu212 cells in vivo.

Effects of HK-II siRNA on Glycolysis in Tu212 Cells Treated with Radiotherapy

To investigate whether HK-II siRNA could reverse the Warburg effect, Tu212 cells were transfected with HK-II

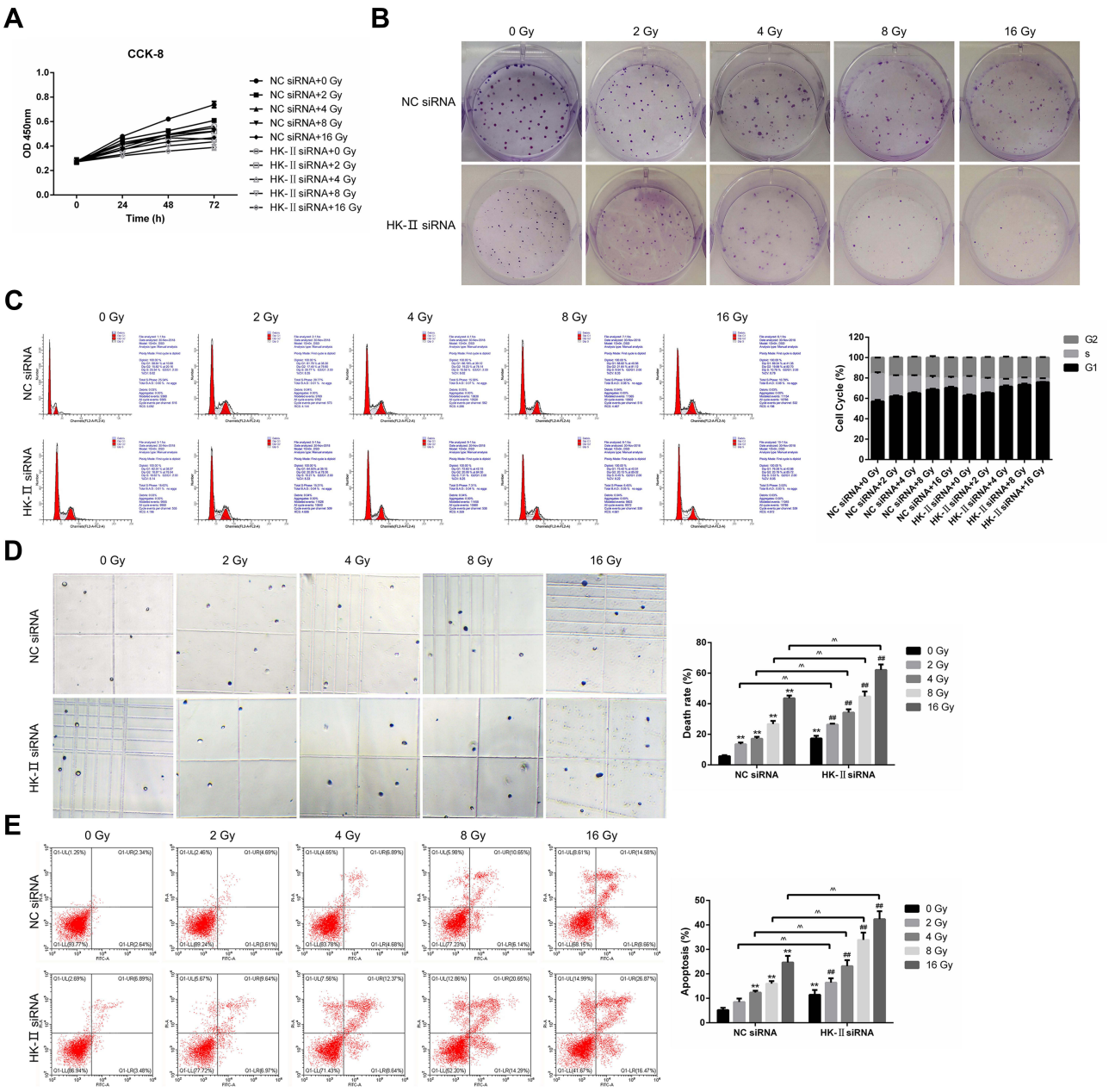

Figure 3 Effects of HK-II siRNA treatment on the viability, cell survival, proliferation, apoptosis, and cell cycle of Tu212 cells. (**A**) Colony formation assay. (**B**) CCK-8 assay. (**C**) Cell cycle. (**D**) Trypan blue staining. (**E**) Apoptosis of Tu212 cells. (**p<0.01, ##p<0.01, and ^^p<0.01).

siRNA, and glucose consumption and lactic acid content were measured after treatment with NC siRNA or HK-II siRNA in the presence or absence of radiotherapy. The data showed that X-ray irradiation at a dose of 2–8 Gy significantly promoted glucose consumption and the secretion of lactic acid from Tu212 cells (all, $P<0.01$), whereas irradiation at 16 Gy had no significant effect on glucose consumption or the secretion of lactic acid (both $P>0.05$) (Figure 6A and B). HK-II siRNA significantly inhibited glucose consumption and the secretion of lactic acid from Tu212 cells (both, $P<0.01$) (Figure 6A and B). The protein expression of enzymes involved in glycolysis was detected using Western blotting. Radiotherapy was shown to enhance the protein expression of PKM2, PFKP, and LD in a concentration-dependent manner from 2 to 8 Gy, whereas irradiation at a dose of 16 Gy had little effect on the expression of these proteins (Figure 6C). HK-II siRNA treatment decreased the expression of PKM2, PFKP, and LD and inhibited the effects of X-ray irradiation on the contents of these proteins (Figure 6C). Similar to the

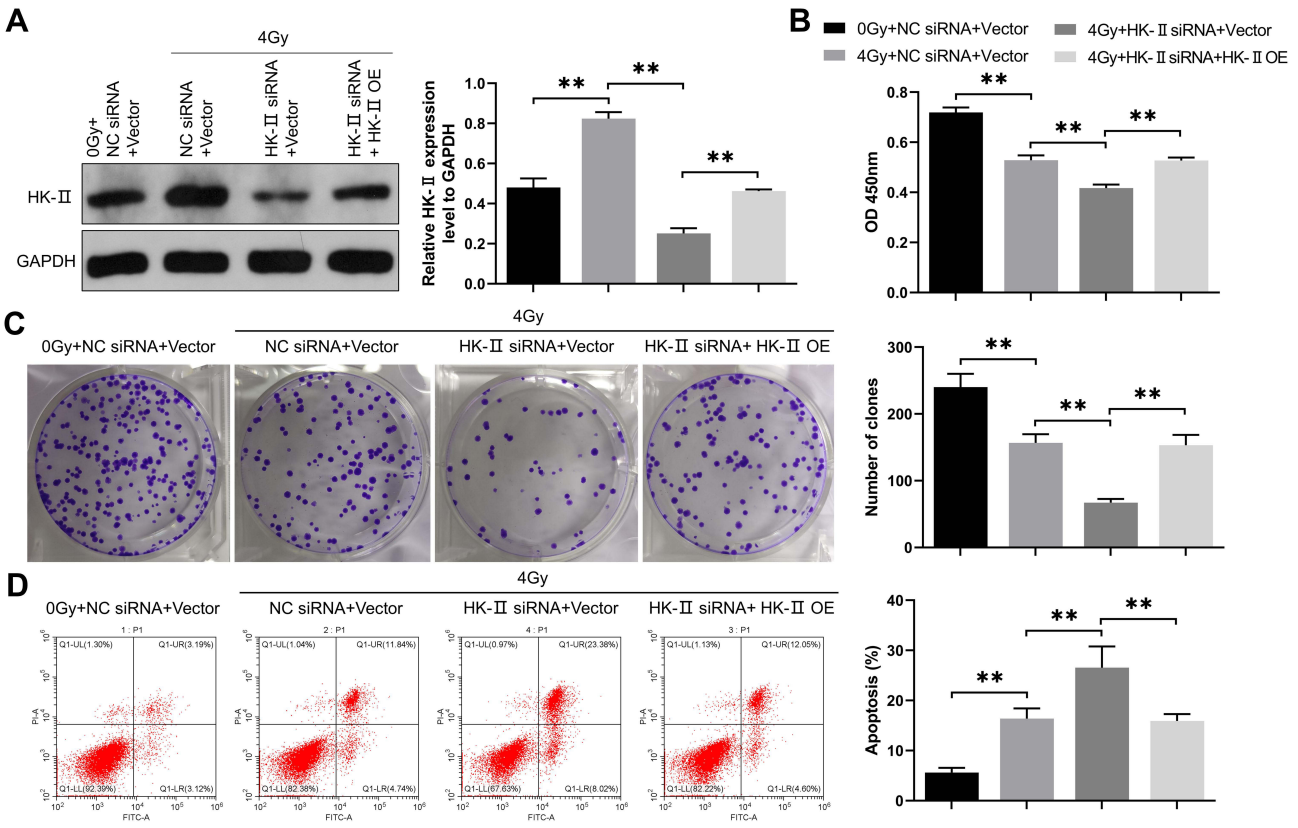

Figure 4 Effects of HK-II overexpression treatment on the viability, cell survival, proliferation, apoptosis, and cell cycle of Tu212 cells with radiotherapy and HK-II siRNA treatment. (**A**) Western blotting results. (**B**) CCK-8 assay. (**C**) Colony formation assay. (**D**) Apoptosis of Tu212 cells. (**p<0.01).

in vitro results, X-ray irradiation promoted the expression of PKM2, PFKP, and LD in xenograft tumors at the protein level (Figure 6), and HK-II siRNA significantly inhibited the protein expression induced by radiotherapy in xenograft tumors (Figure 6D). Therefore, HK-II siRNA treatment inhibited glycolysis induced by X-ray irradiation in Tu212 cells.

Effects of HK-II siRNA on the Tricarboxylic Acid Cycle in Tu212 Cells Treated with Radiotherapy

To investigate whether HK-II siRNA could affect the tricarboxylic acid (TCA) cycle in Tu212 cells, the oxygen consumption rate, ATP content, and expression of enzymes involved in the TCA cycle were examined in Tu212 cells treated with X-rays and/or HK-II siRNA. X-ray irradiation at a dose of 2–8 Gy had a dose-dependent inhibitory effect on the oxygen consumption rate of Tu212 cells, which decreased at 16 Gy. HK-II siRNA treatment increased the oxygen consumption rate of Tu212 cells (Figure 7A). X-ray irradiation significantly reduced the ATP content of Tu212 cells in a dose-dependent manner from 4 to 8 Gy ($P < 0.01$) (Figure 7B), whereas at 16 Gy, X-ray treatment had no significant inhibitory effect on ATP content. HK-II siRNA treatment significantly increased the ATP content of Tu212 cells ($P<0.01$) (Figure 7B). In vitro, X-ray irradiation at a dose of 2–8 Gy upregulated the expression of PDH and CS proteins (all, $P<0.01$) (Figure 7C), whereas X-ray irradiation at 16 Gy had no effect on the expression of these proteins ($P > 0.05$) (Figure 7C). HK-II siRNA treatment significantly suppressed PDH and CS protein expression ($P < 0.01$) (Figure 7C). Similar results were observed in vivo. X-ray irradiation at a dose of 8 Gy increased the expression of PDH and CS proteins in xenograft tumors ($P<0.01$) (Figure 7D), and HK-II siRNA treatment significantly suppressed the upregulated expression of these proteins induced by irradiation at 8 Gy ($P < 0.01$) (Figure 7D). These results suggest that HK-II siRNA treatment partly reversed the inhibitory effect of X-ray irradiation on the TCA cycle, although HK-II siRNA inhibited the expression of PDH and CS induced by X-ray treatment in Tu212 cells.

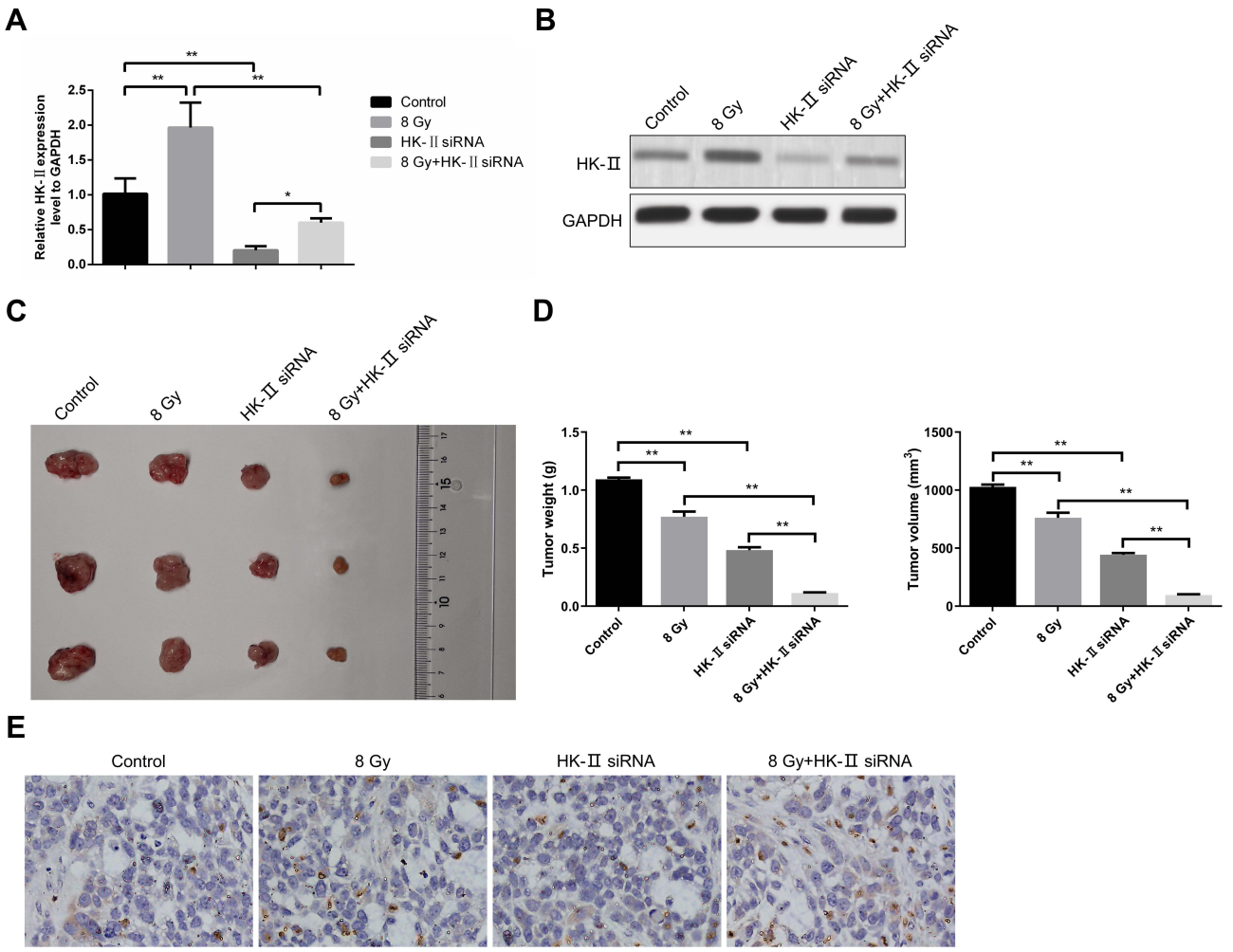

Figure 5 Results of HK-II siRNA treatment in vivo. Expression of (**A**) HK-II mRNA and (**B**) HK-II protein. (**C**) Tumor weight. (**D**) Tumor volume. (**E**) Apoptosis detected via the TUNEL assay. (*p<0.05, and **p<0.01).

Effects of HK-II siRNA on the Expression of VDAC-1 mRNA

We transfected Tu212 cells with HK-II siRNA to investigate whether it had an inhibitory effect on the expression of VDAC-1. X-ray irradiation did not alter the expression of VDAC-1 at the transcriptional or protein level in Tu212 cells in vitro (P>0.05) (Figure 8A and B). HK-II siRNA treatment also had no significant effect on the expression of VDAC-1 in Tu212 cells in comparison to those treated with NC siRNA in the presence or absence of X-ray treatment (Figure 8A and B). Similar to the in vitro results, HK-II siRNA treatment or X-ray irradiation had no significant effect on VDAC-1 mRNA or protein expression in xenograft tumors (P>0.05) (Figure 8C and D). The combination of HK-II siRNA and X-ray irradiation also produced no effect on the expression of VDAC-1 at the mRNA or protein level (Figure 8C and D). These observations indicate that the HK-II siRNA or X-ray treatment had no significant effect on the expression of VDAC-1 in TU212 cells.

Discussion

Although the Warburg effect, ie, aerobic glycolysis, involves incomplete oxidation of glucose leading to the production of lactate and resulting in reduced efficiency of glucose metabolism,[23] it is a major feature of tumor cells and plays an important role in tumor development.[24,25] It has been reported that decreasing glycolysis mediated by the PLK1–Akt regulatory axis could reduce laryngeal squamous cell carcinoma cell viability and inhibit chemoresistance.[26] Glycolysis was shown to be associated with radioresistance in head and neck cancer.[27] HK-II is a key enzyme of

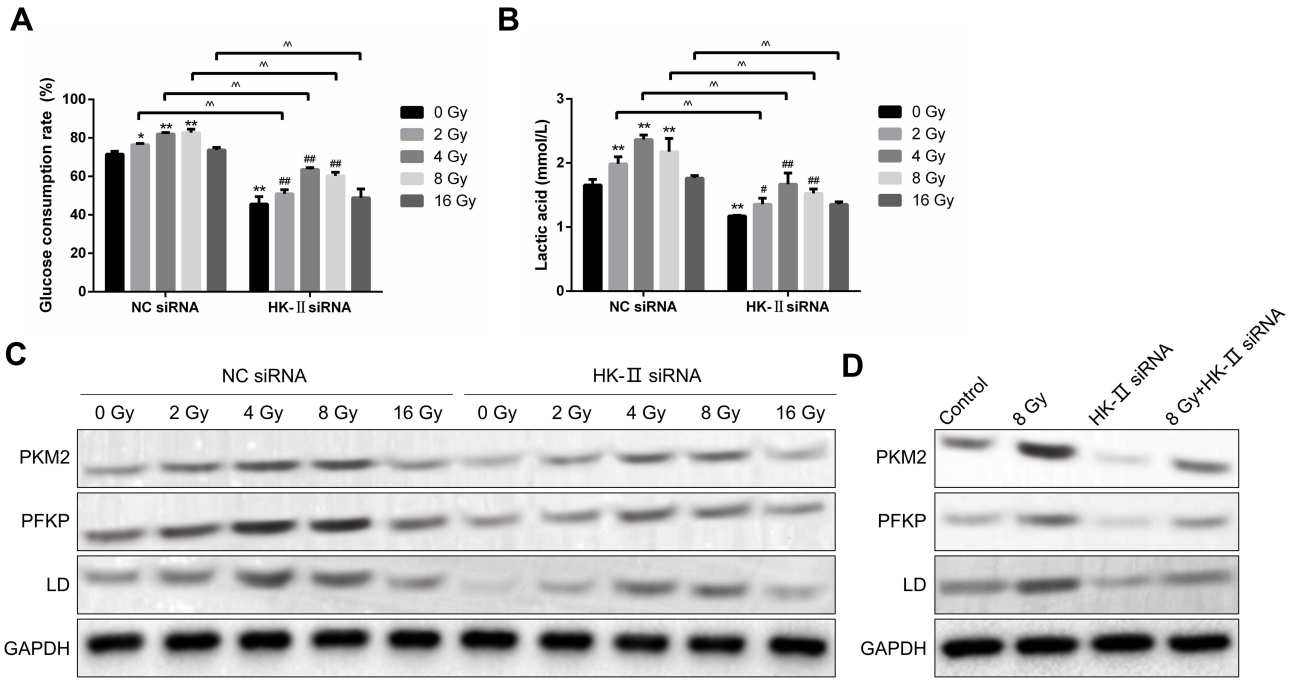

Figure 6 Effects of HK-II siRNA treatment on glycolysis in Tu212 cells treated with radiotherapy. (**A**) Glucose consumption. (**B**) Secretion of lactic acid. (**C**) Expression of PKM2, PFKP, and LD proteins in vitro. (**D**) Expression of PKM2, PFKP, and LD proteins in xenograft tumors. (*p<0.05, **p<0.01, #p<0.05, ##p<0.01, and ^^p<0.01).

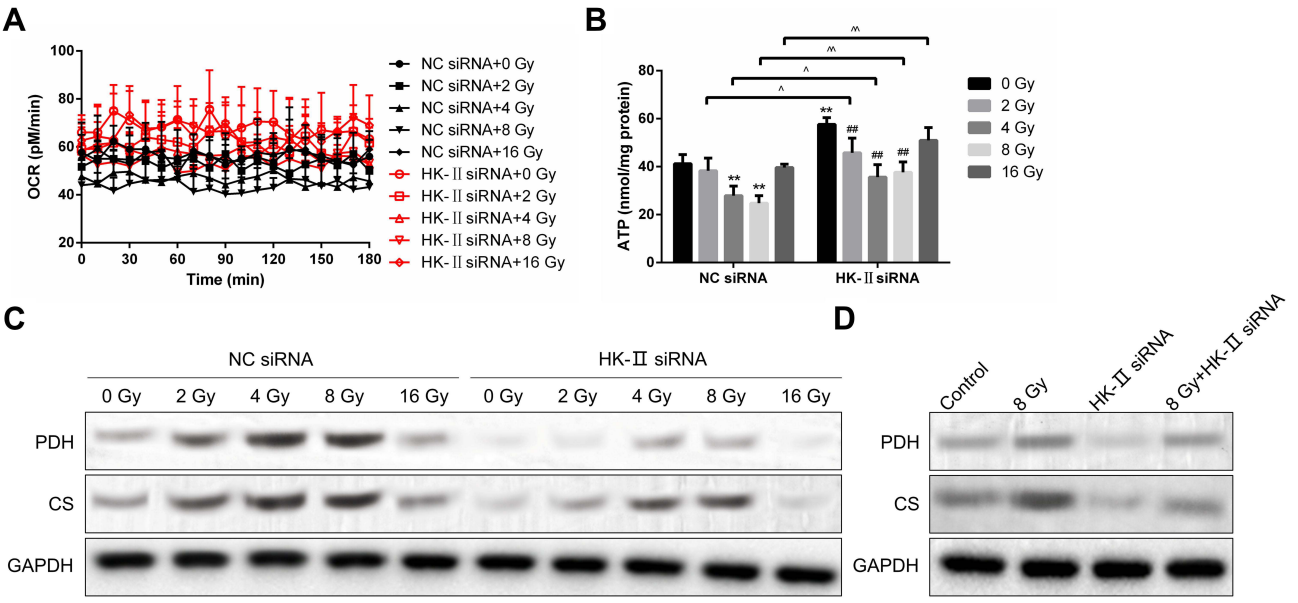

Figure 7 Effects of HK-II siRNA treatment on the TCA cycle in Tu212 cells treated with radiotherapy. (**A**) Oxygen consumption rate. (**B**) ATP content. (**C**) Expression of PDH and CS proteins in vitro. (**D**) Expression of PDH and CS proteins in xenograft tumors. (**p<0.01, ##p<0.01, and ^^compare in pair, ^compare in pairs).

glycolysis[6] and therefore also plays a critical role in the Warburg effect.[6–10] In the present study, we found that the expression of HK-II in laryngeal carcinoma was higher than that in paracarcinoma tissues. Its high-level expression has been shown to be associated with proliferation, growth, invasion, and chemoradioresistance of cancer cells as well as poor prognosis.[6,11–21] The inhibition of HK-II expression may suppress the growth and improve the radiosensitivity of cancer cells.[6,11–21] In nasopharyngeal carcinoma, the targeting of HK-II by hsa-miR-9-5p was shown to improve radiosensitivity.[9,28] HK-II shRNA or metformin could enhance the inhibitory effect of irradiation on the

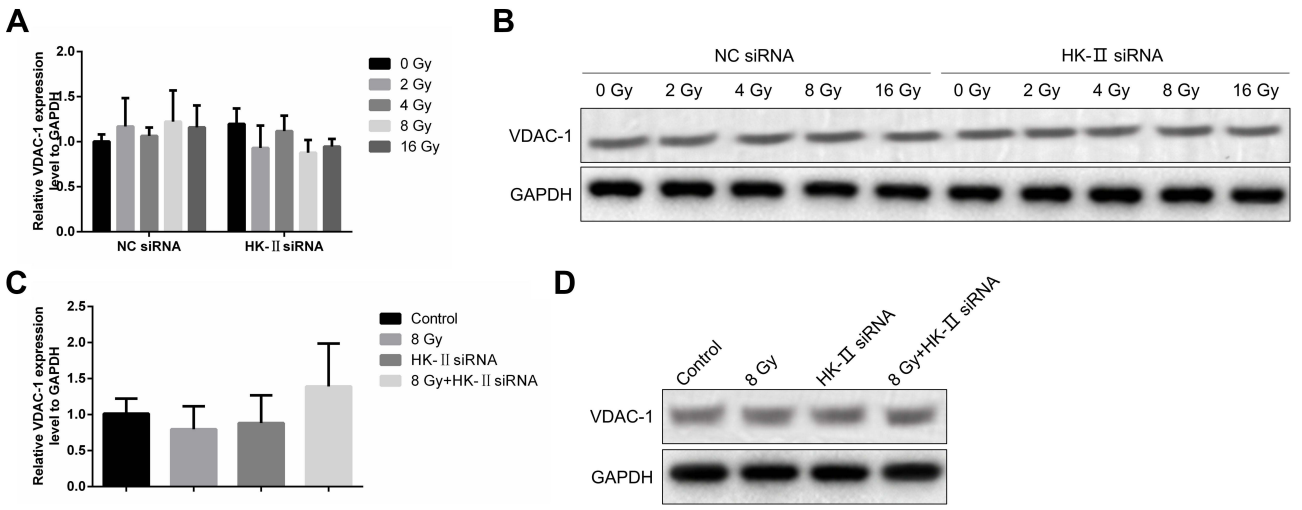

Figure 8 Effects of HK-II siRNA treatment on the expression of HK-II and VDAC-1 mRNA. (**A**) VDAC-1 mRNA expression in vitro. (**B**) VDAC-1 protein expression in vitro. (**C**) VDAC-1 mRNA expression in vivo. (**D**) VDAC-1 protein expression in vivo.

proliferation of cervical carcinoma cells.[29] In glioblastoma, siRNA-mediated inhibition of HK-II increased radiosensitivity by increasing DNA damage.[30] The overexpression of HK-II can increase the proliferation, and decrease apoptosis, of laryngeal carcinoma cells.[19] Gao et al also reported that the inhibition of HK-II suppressed the growth of laryngeal carcinoma cells.[17] The inhibition of HK-II by miR-125a or miR-125b suppressed cell viability and increased apoptosis in laryngeal squamous cell carcinoma cells.[18,20] These results suggest that the targeted inhibition of HK-II may represent a novel therapeutic strategy for laryngeal carcinoma. However, there have been no previous reports regarding the effects of targeted HK-II inhibition on radioresistance in laryngeal carcinoma cells. In the present study, we found that the inhibition of HK-II expression by siRNA significantly increased the effects of X-ray irradiation on the viability, colony formation ability, proliferation, apoptosis, and G2 phase arrest of Tu212 cells in vitro. In vivo, HK-II siRNA significantly enhanced the effects of X-ray irradiation on the growth (weight and volume) of xenograft tumors. These results suggest that the inhibition of HK-II expression by siRNA can sensitize laryngeal carcinoma cells to X-ray irradiation. In the present study, X-ray irradiation at doses of 2–8 Gy upregulated the expression of glycolytic enzymes (HK-II, PKM2, LD, PFKP) and increased both glucose consumption and the lactic acid content in the supernatant, whereas X-ray irradiation at a higher dose of 16 Gy produced no such effects in Tu212 cells. In addition, the inhibition of HK-II by siRNA significantly reduced the expression of other glycolytic enzymes (PKM2, PFKP, and LD), glucose consumption, and the lactic acid content. These findings indicate that X-ray irradiation at doses of 2–8 Gy enhanced glycolysis, resulting in radioresistance, and that the inhibition of HK-II expression with HK-II siRNA can improve radiosensitivity by inhibiting glycolysis in laryngeal carcinoma Tu212 cells.

Glucose metabolism occurs through two mechanisms, ie, glycolysis and oxidative phosphorylation. The pyruvate dehydrogenase complex (PDC) promotes the decomposition of pyruvate into acetyl CoA, which enters the TCA cycle. Pyruvate dehydrogenase kinases (PDKs) inhibit the activity of PDC and block pyruvate decomposition to induce the accumulation of pyruvate in the cytoplasm as well as glycolysis. The inhibition of PDK1 promotes the transition from glycolysis to oxidative phosphorylation and ultimately enhances the radiosensitivity of glioblastoma cells.[31] HK-II plays a critical role in coupling oxidative phosphorylation with glycolysis through the phosphorylation of glucose, using the ATP produced by mitochondria.[31,32] Some studies have suggested that blocking this metabolic coupling may be effective as tumor therapy.[31,33] We found that X-ray irradiation could reduce the oxygen consumption rate of TU212 cells, but it increased the expression of PDH and CS. It has been demonstrated that radiotherapy can reduce the oxygen consumption rate of glioblastoma cells but increase the contents of various TCA cycle-related enzymes, including PDH and CS. We speculate that the expression of other regulatory enzymes involved in the TCA cycle may be decreased although PDH and CS expression are increased,

resulting in no overall enhancement of the TCA cycle. The content and activity of the mitochondrial respiratory chain complex are also closely related to oxidative phosphorylation, and products generated in the TCA cycle can affect oxidative phosphorylation.[34,35] Atovaquone rapidly decreases the oxygen consumption rate of human pharyngeal squamous cell carcinoma FaDu cells by reducing the expression of mitochondrial complex III.[34] The inhibitory effect of papaverine on the oxygen consumption rate in the mouse myeloid breast cancer cell line, EO771, was shown to be dependent on the inhibition of mitochondrial complex I.[35] Consequently, enhancement of the TCA cycle does not indicate enhanced oxidative phosphorylation, and radiotherapy may affect the expression or activity of the mitochondrial respiratory chain complex, thereby inhibiting oxidative phosphorylation in Tu212 cells. In the present study, HK-II siRNA was also shown to inhibit radiotherapy-induced increases in PDH and CS expression as well as the radiation-induced decrease in oxygen consumption. HK-II siRNA could inhibit the radiation-induced downregulation of oxidative phosphorylation, independent of changes in PDH and CS expression. Further studies are required to determine whether oxidative phosphorylation plays a regulatory role in radioresistance in laryngeal carcinoma and HK-II siRNA-induced radiosensitivity.

Interestingly, irradiation at 2–8 Gy reduced the ATP content, whereas HK-II siRNA treatment increased it. The ATP content generated from oxidative phosphorylation was higher than that generated from glycolysis, and therefore, there was no decrease in total amount of ATP. These findings suggest that the inhibition of HK-II by siRNA can only partially reverse oxidative phosphorylation. The mechanism underlying the enhancement of the radiosensitivity of Tu212 cells through the inhibition of HK-II by siRNA is independent of the ATP pathway. Lactate generated by glycolysis plays an important role in tumor progression. Lactate was shown to promote tumor cell growth and immune escape through macrophage polarization as well as regulate T cell and NK cell function.[36,37] Lactate can also enhance the metastatic ability of cervical cancer cells by regulating the level of miR-744.[38] Further studies are required to determine whether the mechanism underlying the HK-II siRNA-induced radiosensitivity of laryngeal carcinoma is related to the blockade of lactic acid production.

HK-II binds to VDACs in mitochondria, and the formation of the VDAC-1 and HK-2 complex modulates the integrity and permeability of the mitochondrial membrane. With a decrease in the level of HK-II in mitochondria, the interaction between HK-II and VDACs would be disrupted, resulting in a marked increase in membrane permeability and the release of proapoptotic enzymes such as cytochrome C. In the present study, X-ray irradiation and the inhibition of HK-II by siRNA did not affect the expression of VDAC-1 at the mRNA or protein level. The mechanism underlying the enhancement of radiosensitivity of Tu212 cells through the inhibition of HK-II by siRNA may be independent of changes in the expression of VDAC-1. Further investigation is required to determine whether the inhibitory effect of HK-II siRNA on radioresistance in laryngeal carcinoma cells is dependent on the VDAC-1 pathway.

In conclusion, the results of the present study showed that the inhibition of HK-II by siRNA could enhance the effects of radiotherapy on cell proliferation and apoptosis in Tu212 cells. This might have been mediated through the inhibition of glycolysis and partial reversion of oxidative phosphorylation in laryngeal carcinoma Tu212 cells in vitro and in vivo. Targeting HK-II may be an important therapeutic strategy to improve the radiosensitivity of laryngeal carcinoma and enhance the survival of patients with laryngeal carcinoma.

Data Sharing Statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics Approval and Informed Consent

This study was conducted under the guidelines and with the approval of Second Hospital of Jiaxing City, Jiaxing City, Zhejiang Province, 314000, China (No. jxey-2017013).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or

critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests in this work.

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