Inhibition of microRNA-155 sensitizes lung cancer cells to irradiation via suppression of HK2-modulated glucose metabolism

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Abstract. MicroRNAs (miRNAs) are small non-coding regulatory RNAs, which are involved in the post-transcriptional regulation of gene expression. miRNA (miR)-155, which has previously been reported to be overexpressed in lung cancer, is correlated with poor patient prognosis. The present study aimed to investigate the effects of miR-155 on the radiosensitivity of human non-small cell lung cancer (NSCLC) cells. To explore the roles of miRNAs in the regulation of irradiation sensitivity of human lung cancer cells, the expressions of miR-155 in response to irradiation, have been studied by RT-qPCR, and the putative direct target of miR-155 was identified by western blot and luciferase assays. The results of the present study revealed that the expression of miR-155 was induced by irradiation, thus suggesting a positive correlation between miR-155 and radiosensitivity. Furthermore, overexpression of miR-155 rendered lung cancer cells resistant to irradiation. In addition, hexokinase 2 (HK2) was identified as an indirect target of miR-155; exogenous overexpression of miR-155 upregulated the expression of HK2, whereas inhibition of miR-155 by antisense miRNA suppressed HK2 expression. In addition, HK2-modulated glucose metabolism was significantly upregulated by overexpression of miR-155. Notably, inhibition of miR-155 sensitized lung cancer cells to irradiation via suppression of glucose metabolism. In conclusion, the present study reported a novel function for miR-155 in the regulation of NSCLC cell radiosensitivity, thus suggesting that miR-155 may be considered a therapeutic target for the development of anticancer drugs.

Introduction

MicroRNAs (miRNAs) are small non-coding regulatory RNAs 17-25 nucleotides long, which are involved in the post-transcriptional regulation of gene expression (1). More than 50% of miRNA genes are located in cancer-associated genomic regions or fragile sites, thus suggesting that miRNAs have important roles in tumor development processes, including apoptosis, invasion, metastasis, proliferation and drug resistance (2). Usually, miRNAs that lead to tumorigenesis are classed as oncomiRs, whereas miRNAs whose functional loss can contribute to the malignant transformation of normal cells, are classed as tumor suppressors (3). miRNA (miR)-155 is one of the most commonly upregulated miRNAs in several types of cancer, including non-small cell lung cancer (NSCLC) (4), breast cancer (5), pancreatic cancer (6), colon cancer (7), renal cancer (8), Hodgkin and B cell lymphoma (9), and secondary acute leukemia (10). Therefore, the dysregulated expression of miR-155 may be an important target for the diagnosis, prognosis and treatment of cancer (11). However, the specific mechanism of action of miR-155 in cancer is currently only partially known.

NSCLC represents the most frequent type of lung cancer, and has a 5-year overall survival rate of <15% (12). At present, radiation therapy is regarded as an effective treatment strategy for NSCLC, which uses high-energy rays or particles to destroy lung cancer cells (13). However, radioresistance remains a major barrier that limits the efficacy of radiotherapy (13). Therefore, the development of novel approaches is urgently required, in order to overcome the radioresistance of NSCLC and improve the survival rate of patients. A previous study reported that under hypoxic conditions, miR-155 expression was induced, leading to increased resistance of NSCLC cells to irradiation (14).

The majority of cancer cells exhibit increased glycolysis and dysregulated mitochondrial function, in order to provide sufficient energy for proliferation (15); this unique feature of cancer cells is known as the Warburg effect. Hexokinases (HKs) catalyze the first committed step in glucose metabolism by catalyzing the phosphorylation of glucose to glucose-6-phosphate (G6P). Therefore, hexokinases influence the direction of glucose flux within cells, which is tightly correlated with the processes of tumor initiation and maintenance (15). In addition, elevated glycolysis of cancer cells has been shown to contribute to chemo- and radiotherapy resistance (16). It has previously been reported that radiation induces aerobic glycolysis via reactive oxygen species (17). Furthermore, the oncogene Akt has been shown to promote aerobic glycolysis, which renders cancer cells resistant to irradiation (18), thus
indicating that glycolysis may be a target for the development of anticancer therapies. The present study investigated the role of miR-155 in the radiosensitivity of NSCLC. The results revealed that inhibition of miR-155 may sensitize NSCLC cells to radiation, thus suggesting that miR-155 may be considered a therapeutic target for the development of anticancer drugs.

Materials and methods

Cells and cell culture. The A549 and H460 NSCLC cell lines were purchased from American Type Culture Collection (Manassas, VA, USA), and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All of the cells were cultured at 37˚C in a humidified incubator containing 95% air and 5% CO₂, and A549 and H460 cells were treated with 3BrPA at 50 µM for 24 h.

Antibody and reagents. The following antibodies were used in the present study: Anti-β-actin (cat. no. 4967; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-hexokinase 2 (HK2) (cat. no. 2106; Cell Signaling Technology, Inc.). 3-Bromopyruvate (3BrPA) was purchased from Sigma-Aldrich China, Inc. (Hong-Kong, China). The activities of HK were measured using the Hexokinase Colorimetric Assay kit (Sigma-Aldrich China, Inc.).

Ionizing radiation treatment. Approximately 5x10⁴ cells per 6 cm-dish were exposed to various doses of irradiation (0.2, 0.4, 0.5, 0.6, 1, 1.5, 2, 2.5, 3, 4 and 5 Gy) at room temperature using a Cs-137 irradiator (HWM D-2000; Siemens AG, Munich, Germany) at a dose rate of 2 Gy/min. The cells were then trypsinized, re-plated in a cell culture dish, and incubated for 16 h, prior to downstream analysis.

Plasmid DNA and miRNA transfections. Transfection was performed using the Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. Briefly, 0.5-1x10⁴ cells/well were plated onto a 6-well plate, and were incubated overnight until they had reached 70-90% confluence. The next day, plasmid DNA (4 µg; Addgene, Inc., Cambridge, MA, USA), pre-miR-155 (100 nM) or anti-miR-155 (100 nM) was diluted in Opti-MEM I reduced serum medium (Invitrogen; Thermo Fisher Scientific, Inc.) and was added to each well. The cells were then incubated for a further 48 h. Cell viability was measured using the MTT assay, according to the manufacturer's instructions. Briefly, an equal number of cells were plated into 96-well plates with culture medium containing either 3BrPA or phosphate-buffered saline (PBS; Sigma-Aldrich China, Inc.) for the untreated control. Following treatments, 0.1 mg/ml MTT was added to each well and was incubated at 37˚C for 4 h. Plates were centrifuged at 450 x g for 5 min at room temperature, and the medium was discarded. Dimethyl sulfoxide (0.15 ml; Sigma-Aldrich China, Inc.) was added to each well to solubilize the crystals. Absorbance was measured spectrophotometrically at a wavelength of 570 nm using an ELx800 universal microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

dcDNA preparation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted following homogenization of the cells using the RNeasy Mini kit (Qiagen Sciences, Inc., Germantown, Maryland MD). DNase digestion was performed during the RNA extraction using the RNase free DNase set (Qiagen, Inc., Valencia, CA, USA). Total RNA (1 µg) was reverse transcribed using the TaqMan microRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and TaqMan microRNA Assays kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The precursor miR-155 and RNU6B (served as an internal control) primers were used as follows: miR-155, F 5’-CTGAGGTATTCGCAATATTTCCACAGA-3’ and R 5’-ATGCGCCGGCCCTGAAGTGGTATGACAAAACTC-3’; RNU6B, F 5’-CTGGTATTCGCAATATTTCCACAGA-3’ and R 5’-ATGCGCCGGCCCTGAAGTGGTATGACAAAACTC-3’. Total RNA (1 µg) was reverse transcribed using the TaqMan microRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and TaqMan microRNA Assays kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The precursor miR-155 and RNU6B (served as an internal control) primers were used as follows: miR-155, F 5’-CTGAGGTATTCGCAATATTTCCACAGA-3’ and R 5’-ATGCGCCGGCCCTGAAGTGGTATGACAAAACTC-3’; RNU6B, F 5’-CTGGTATTCGCAATATTTCCACAGA-3’ and R 5’-ATGCGCCGGCCCTGAAGTGGTATGACAAAACTC-3’.

Western blot analysis. Cells were removed from the treated or non-treated conditions, and were immediately placed on ice. After rinsing with phosphate-buffered saline, the cells were

HK activity assay. The cells were seeded onto a 6-well plate at a density of 3x10⁴/well overnight. The cells were then collected and HK activities were measured using the Hexokinase Colorimetric Assay kit (Sigma-Aldrich China, Inc.), according to the manufacturer's protocol.

Glucose consumption and lactate production. The cells were seeded onto a 6-well plate at a density of 3x10⁴/well, and the culture medium was replaced with low glucose DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) after 6 h. The concentrations of glucose and L-lactate were measured after a further 24 h using a Glucose Test kit (Applygen Technologies, Inc., Beijing, China) and an L-lactate Assay kit (Eton Bioscience, Inc., San Diego, CA, USA), respectively, according to the manufacturer's instructions. Cell viability assay. A total of 1x10⁴ cells/well were seeded onto 48-well plates overnight. The medium was subsequently replaced with fresh medium with or without 3BrPA at the indicated concentrations, and the cells were incubated for a further 48 h. Cell viability was measured using the MTT assay, according to the manufacturer's instructions. Briefly, an equal number of cells were plated into 96-well plates with culture medium containing either 3BrPA or phosphate-buffered saline (PBS; Sigma-Aldrich China, Inc.) for the untreated control. Following treatments, 0.1 mg/ml MTT was added to each well and was incubated at 37˚C for 4 h. Plates were centrifuged at 450 x g for 5 min at room temperature, and the medium was discarded. Dimethyl sulfoxide (0.15 ml; Sigma-Aldrich China, Inc.) was added to each well to solubilize the crystals. Absorbance was measured spectrophotometrically at a wavelength of 570 nm using an ELx800 universal microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).
scraped, collected and protein was extracted from them using lysis buffer (Thermo Fisher Scientific, Inc.). Protein concentration was quantified using the Bradford reagent (Sigma-Aldrich China, Inc.) according to the manufacturer's instructions. Total protein (50 µg/each lane) was loaded and size fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and was transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific, Inc.). The membranes were incubated with a blocking reagent of 5% non-fat milk in PBS-Tween 20 (PBST), washed with PBST and probed with primary antibodies (1:1,000) at 4˚C overnight. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 2 h at room temperature, followed by detection with a Super Signal Enhanced Chemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL, USA). For sequential blotting, the membranes were stripped with Stripping Buffer (Pierce Biotechnology, Inc.) and re-probed with appropriate antibodies.

Figure 1. miR-155 is modulated by irradiation in NSCLC cells. (A) A549 (left) and H460 (right) human NSCLC cell lines were treated with irradiation at 0.2, 0.4 and 0.6 Gy, and the expression levels of miR-155 were measured "P<0.01 and ""P<0.001 vs. the non-treatment group. (B) A549 (left) and H460 (right) cells were transiently transfected with pre-miR-155 or control miR for 48 h, and the survival curve was measured upon exposure to the indicated doses of irradiation. *P<0.05 and **P<0.01 vs. the control miR group. Data are presented as the mean ± standard error of the mean. miR, microRNA; NSCLC, non-small cell lung cancer.

Figure 2. Glucose metabolism of lung cancer cells was upregulated by miR-155. A549 (left) and H460 (right) cells were transfected with pre-miR-155, anti-miR-155 or control miRNA for 48 h and (A) glucose uptake and (B) lactate production were measured. Data are presented as the mean ± standard error of the mean. *P<0.05; **P<0.01. miR, microRNA.
Results

miR-155 is induced by radiation and correlated with radiation resistance. It has previously been reported that miR-155 levels are increased in numerous types of cancer, including NSCLC, thus suggesting that miR-155 may act as an oncomiR (4). The present study examined the role of miR-155 during the irradiation of cancer cells. Two NSCLC cell lines: A549 and H460, were exposed to numerous non-toxic doses of radiation. Notably, the expression levels of miR-155 were significantly upregulated by irradiation, indicating that miR-155 may have an important role in cancer radiotherapy (Fig 1A). In addition, the present study determined whether miR-155 was able to influence the viability of lung cancer cells in response to radiotherapy. A549 and H460 cells were transiently transfected with a miR-155 precursor; overexpression of miR-155 in lung cancer cells had a radioprotective effect on A549 and H460 cells (Fig. 1B), as compared with in cells transfected with control miRNA. The half maximal inhibitory concentration of control A549 or H460 cells in response to radiotherapy was ~2 Gy, which was lower than that of the cells overexpressing miR-155 (5 Gy). These results indicate a positive correlation between miR-155 and radioresistance in NSCLC cells.

miR-155 upregulates the expression of HK2. HK2 is a key enzyme of glycolysis, which catalyzes the formation of glucose-6-phosphate from glucose (20). HK2 activity is positively correlated with the rate of glycolysis. In the present study, HK2 was identified as an indirect target of miR-155 in NSCLC cells (Fig. 3A and B). Overexpression of miR-155 significantly upregulated HK2 protein expression in A549 and H460 cells. Conversely, inhibition of miR-155 downregulated HK2 expression. These results suggest a putative mechanism for the acquired radioresistance of NSCLC cells that overexpress miR-155.

Suppression of glycolysis by HK2 inhibitor sensitizes NSCLC cells to radiation. To investigate whether miR-155-induced glycolysis was due to the upregulation of HK2, lung cancer cells were treated with 3BrPA, which is a specific inhibitor of HK2. Treatment with 3BrPA significantly suppressed the HK activities of A549 and H460 cells (Fig. 4A). Furthermore, the glucose consumption and lactate production of A549 and H460 cells were decreased following treatment with 3BrPA (50 µM) for 24 h (Fig. 4B and C). Subsequently, the present study examined whether inhibition of HK2 by 3BrPA was able to sensitize lung cancer cells to radiation. A549 cells were treated with 3BrPA (50 µM) for 24 h, and were then exposed to various doses of radiation. As expected, A549 cells regained sensitivity to irradiation following treatment with 3BrPA (Fig. 4D). These results suggest that suppression of glycolysis by inhibiting HK2 may be considered a therapeutic strategy to enhance the efficacy of radiotherapy.
Inhibition of miR-155 elevates cancer cell sensitivity to radiation via the suppression of glycolysis. The results of the present study revealed that miR-155 upregulated HK2 expression, which contributed to radioresistance. Subsequently, the radiosensitivity of lung cancer cells was determined in response to various doses of radiation, with or without inhibition of miR-155. As shown in Fig. 5A, inhibition of miR-155 rendered A549 cells more sensitive to irradiation, as compared with the cells transfected with control miRNA. These results are consistent with the previous results that A549 cells are more sensitive to irradiation following treatment with 3BrPA. To determine whether inhibition of miR-155 sensitized lung cancer cells to irradiation via the suppression of glycolysis, anti-miR-155 was transfected into A549 cells undergoing radiation and glucose metabolism was measured. As shown in Fig. 5B, irradiation induced glycolysis, which is consistent with the results of a previous study (17). In addition, under irradiation, inhibition of miR-155 significantly suppressed glucose metabolism (Fig. 5B), thus suggesting that miR-155-mediated radiosensitivity may be associated with the modulation of glycolysis in lung cancer cells.

Restoration of HK2 renders miR-155-inhibited lung cancer cells resistant to irradiation. To further verify the results of the present study, an overexpression vector containing wild type HK2 and antisense miR-155 were co-transfected into A549 cells undergoing radiation, control cells were transfected with a control vector (Fig. 5C). As expected, restoring the expression of HK2 recovered the radioresistance of A549 cells in response to radiation (Fig. 5D). These results clearly
indicate that HK2 has an important role in lung cancer cell irradiation sensitivity, and overexpression of miR-155 confers radioresistance at least partially via the indirect upregulation of HK2 expression.

Discussion

The present study observed that radiation induces the expression of miR-155, which promotes glucose metabolism via the indirect upregulation of HK2. It has previously been reported that miR-155 functions as an important oncomiR, which is a commonly upregulated miRNA in solid and hematological malignancies, including lung cancer, and is correlated with poor patient prognosis (11). The present study demonstrated that miR-155 is induced by radiation, and overexpression of miR-155 in lung cancer cells contributes to radioresistance. In addition, forced inhibition of miR-155 by antisense transfection may render lung cancer cells sensitive to radiation. These results suggested that miR-155 may be correlated with radioresistance, and support the oncogenic roles of miR-155 in NSCLC.

The present study identified an essential role for the miR-155-HK2-glycolysis axis in the Warburg effect. Compared with normal cells, cancer cells exhibit a metabolic switch from oxidative metabolism to anaerobic glycolysis, even in the presence of oxygen. This unique feature of cancer cells is known as the Warburg effect. Activation of the glycolytic pathway is frequently observed in radioresistant cancer cells (17,18). Furthermore, disruption of glycolysis has been considered a possible target for anticancer therapy. It has previously been reported that chemo- and radioresistant cancer cells display an elevated anaerobic glycolysis rate, as compared with parental cells (16), thus indicating that dysregulated glycolysis is correlated with chemo- and radioresistance. At present, whether activation of glycolysis is involved in miRNA regulation remains largely unknown. The present study reported that elevated glycolysis in lung cancer cells by the exogenous expression of miR-155 is correlated with radioresistance. Lung
cancer cells become increasingly resistant to radiation with increased glycolysis, indicating that dysregulated glycolysis may be a target for overcoming resistance.

Notably, miR-155 was shown to exert its role in glycolysis via upregulation of HK2, which is a key glycolytic enzyme responsible for catalyzing the irreversible first step of glucose metabolism. It has previously been reported that HK2 has an important role in controlling tumorigenesis and chemoresistance (21). Although the indirect regulation of HK2 by the miR-155-miR-143 cluster has been described previously (22), the present study investigated the novel roles of miR-155-mediated glycolysis in radiation resistance via the upregulation of HK2. In our next project, we will focus on the mechanisms underlying radiation-induced miR-155 expression, and screen more differentially regulated miRNAs involved in the radiosensitivity of lung cancer cells. In conclusion, the results of the present study highlight the importance of miR-155 in regulating lung cancer radiosensitivity, and may provide potential targets for the development of cancer therapeutic strategies.

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References