MicroRNA-148b enhances the radiosensitivity of non-Hodgkin’s Lymphoma cells by promoting radiation-induced apoptosis

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Growing evidence has demonstrated that microRNAs (miRNAs) play an important role in regulating cellular radiosensitivity. This study aimed to explore the role of miRNAs in non-Hodgkin’s lymphoma (NHL) radiosensitivity. Microarray was employed to compare the miRNA expression profiles in B cell lymphoma cell line Raji before and after a 2-Gy dose of radiation. A total of 20 differentially expressed miRNAs were identified including 10 up-regulated and 10 down-regulated (defined as $P < 0.05$). Among the differentially expressed miRNAs, miR-148b was up-regulated 1.53-fold in response to radiation treatment. A quantitative real-time polymerase chain reaction (PCR) assay confirmed the up-regulation of miR-148b after radiation. Transient transfection experiments showed that miR-148b mimic could inhibit the proliferation of Raji cells and down-regulated by miR-148b inhibitor in the Raji cells. A proliferation assay showed that miR-148b could inhibit the proliferation of Raji cells before and after radiation. A clonogenic assay demonstrated that miR-148b sensitized Raji cells to radiotherapy. MiR-148b did not affect the cell cycle profile of post-radiation Raji cells compared with controls. An apoptosis assay showed that miR-148b enhanced apoptosis of Raji cells after irradiation. Taken together, these results demonstrate that miR-148b increased the radiosensitivity of NHL cells probably by promoting radiation-induced apoptosis, which suggests that miR-148b plays an important role in the response of NHL to ionizing radiation.

Keywords: miR-148b; ionizing radiation; non-Hodgkin’s lymphoma; Raji cell; apoptosis

INTRODUCTION

Non-Hodgkin’s lymphoma (NHL) is a heterogeneous group of lymphoid malignancies characterized by an abnormal clonal proliferation of B cells, T cells or both. The incidence of NHL has increased strikingly during the last four decades, nearly doubling from 1970 to 1990. The increased incidence of NHL has been partly attributed to recent advances in molecular diagnostic techniques, the aging of the population, the acquired immune deficiency syndrome (AIDS) epidemic, other infectious agents and occupational exposures [1]. Radiotherapy is a well-established approach in the management of NHL across different histological and clinical sub-types. In indolent NHL, radiotherapy may cure early stage disease and palliate more advanced diseases [2, 3]. In aggressive NHL, radiotherapy is used in stage I disease with curative intent after a short course of chemotherapy, and may also be used to consolidate chemotherapy response in bulk or extra-nodal disease [4, 5]. NHLs are known to be radiosensitive tumors. However, for diffuse histiocytic lymphoma, local failure rates were in the range of 20–30% regardless of the radiation dose.
delivered [6]. These data suggest a subset of resistant disease in the range of 20%. Eliminating these radiation-resistant cells may be a key to improving lymphoma radiotherapy. Therefore, it is important to develop new drugs to decrease radio-resistance or increase radiosensitivity in these lymphomas. In fact, rituximab and imexon could efficiently enhance the radiosensitivity of NHL cells [7–10].

MicroRNAs (miRNAs) are small, 20–22-nucleotide-long members of the non-protein-coding RNA family. Following binding to partially complementary sites, usually in the 3' untranslated regions (UTRs) of mRNAs, miRNAs cause an inhibition of translation and some degree of degradation of the target mRNA [11]. Therefore, miRNAs are negative regulators of the expression of over 30% of mRNAs and play important roles in fundamental processes such as development, differentiation, cell proliferation, apoptosis and stress responses [12].

Notably, accumulating evidence indicates that miRNAs are implicated in cell radiosensitivity. Weidhaas et al. found that the let-7 family of miRNAs was overrepresented in a class of miRNAs exhibiting altered expression in response to radiation, and functioned to increase radiosensitivity [13]. Oh et al. found that the overexpression of let-7a decreased K-Ras expression and radiosensitized A549 cells [14]. MiR-521 modulated the expression of DNA repair protein CSA and sensitized prostate cancer cells to radiation treatment [15]. Transient overexpression of miR-181a significantly sensitized malignant glioma cells to radiation treatment concurrent with the down-regulation of the protein Bcl-2 [16]. Furthermore, miR-221 and miR-222 regulated radiosensitivity, cell growth and invasion of human gastric cancer cells, possibly via direct modulation of PTEN expression [17]. Therefore, miRNAs emerge as new targets for improving cancer radiotherapy.

The purpose of this study was to investigate the role of miRNAs in the regulation of NHL radiosensitivity. The miRNA expression profiles of B cell lymphoma cell line Raji before and after irradiation were examined by microarrays. The expression of miR-148b was significantly up-regulated after irradiation in Raji cells, which might regulate the radiosensitivity of lymphoma. Subsequent functional experiments confirmed that miR-148b could regulate the radiosensitivity of Raji cells.

**MATERIALS AND METHODS**

**Cell culture**

The human NHL cell lines Raji and RL were obtained from ATCC and cultured in RPMI-1640 medium (Hyclone, USA) supplemented with 10% (v/v) fetal bovine serum (TBD, Tianjing, China), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, USA) in a humidified atmosphere containing 5% CO₂ at 37°C. Exponentially growing cells were used for all experiments.

**Irradiation planning and delivery**

External beam radiation was delivered by an Elekta Precise Linear Accelerator (Elekta Oncology Systems, UK) with a 6-MV photon beam. A 40 × 40 cm field size was utilized and Petr dishes were placed 100 cm from the source on 1.5 cm of superflab bolus. Monitor units (MUs) were calculated to deliver the dose to a depth of d_{max} at a dose rate of 2.5 Gy/min. Control cells that were not irradiated were removed from the incubator and transported to the radiation site for the period of irradiation of the other cells.

**MiRNA microarray analysis**

At room temperature, about 6 × 10⁶ cells were irradiated with the appropriate radiation dose, which produced 50–60% inhibition of growth in Raji cells. The irradiated and control cells were then maintained for 4 h. Total RNA was harvested using TRizol (Invitrogen, USA) and RNase mini kit (QIAGEN, Germany) according to the manufacturers’ instructions. RNA samples were measured by Nanodrop, then labeled using the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark) and hybridized on the miRCURY™ LNA Array (v.14.0, Exiqon) [18]. The Genepix 4000B scanner (Axon Instruments, Union City, CA, USA) was used to acquire the microarray images. Data were analyzed with Genepix Pro 6.0 (Axon Instruments).

**Quantitative real-time PCR (qRT-PCR) analysis for miRNA**

Cell irradiation and RNA preparation were as described above. Expression of mature miRNA was detected using stem-loop reverse transcription followed by real-time PCR analysis [19]. cDNA was synthesized according to the protocol of the reverse transcription system (Epicentre, Madison, WI, USA). The SYBR and U6 genes were used for detecting gene amplification and normalization, respectively. The primers used for stem-loop qRT-PCR for miR-148b and U6 snRNA are listed in Tables 1 and 2. The real-time PCR reactions were performed on a Rotor-Gene 3000 Real-Time PCR cycler (Corbett Research, Australia), according to the manufacturer’s instructions. The fold-change of each miRNA in post-irradiation cells relative to the control was calculated using the 2^{−\Delta\Delta Ct} Method [20], where ΔΔCt = ΔCt cells 4 h post-irradiation – ΔCt cells and ΔCt = Ct miRNA – Ct U6 snRNA.

**Transient transfection of cells with miR-148b mimic or inhibitor**

Cells were plated in 12-well plates (3 × 10⁵ cells/well) and transfected with duplex oligonucleotide (mimic, 50 nM) or single antisense of miR-148b (inhibitor, 100 nM) (Ribobio, China) using Entranster™ R transfection reagent (Engreen Biosystem, Beijing, China) according to the manufacturer’s
instructions. Control mimic (50 nM) or control inhibitor (100 nM) (Ribobio) was used as a control. After transfection for 24 h, the cells were harvested for further experiments. A 5'-Cy3 negative control miRNA (Ribobio) was used for the measurement of transfection efficiency.

**Cell proliferation assay**

Cells were seeded into 96-well culture plates at a density of 3000 cells/well. After irradiation, the cells were cultured in a humidified chamber at 37°C. Each day for four consecutive days, viable cells were evaluated with the CCK-8 Assay (Dojindo, Japan) according to the manufacturer’s instructions. CCK-8 solution was added to the cells in 96-well plates and the plates were incubated at 37°C for 4 h, and the optical density of each well was read at 450 nm using a microplate reader (ELX800, Bio-Tek, USA).

**Clonogenic assay**

Five hundred cells were plated in 24-well plates in 1 ml of Iscove’s Modified Dulbecco’s Medium (IMDM) containing 1% methylcellulose (Sigma, USA), 10% bovine serum albumin and 10% fetal bovine serum and cultured for 4 h, and then the cells were irradiated with 0, 1, 2, 3 and 4 Gy. Cultures were incubated at 37°C in a fully humidified atmosphere with 5% CO2 and scored on Day 8 under an inverted microscope (Leica, Wetzlar, Germany). Only colonies containing >50 cells were counted. The surviving fraction was calculated as a ratio of the number of colonies formed divided by the total number of cells plated times the plating efficiency. The surviving fraction was further plotted in log scale.

**Cell cycle analysis**

Cells were prepared for fluorescence activated cell sorting (FACS) to assess the relative distribution in their respective phase of the cell cycle. Cells were transfected with miR-148b mimic, inhibitor or controls and cultured for 48 h, and the cells were collected by centrifugation, washed in phosphate buffered saline (PBS) and fixed overnight at 4°C in 70% ethanol. After being washed twice with PBS, DNA was stained with propidium iodide (50 μg/ml) in the presence of 1 mg/ml of RNase A for 30 min. Analysis was performed on a BD FACSCanto flow cytometer (BD Biosciences, USA). At least 10 000 events were acquired using ModFit LT V3.0 software (BD Biosciences, USA).

**Annexin V-PI apoptosis assay**

Approximately 5 × 10⁵ cells were transfected with miR-148b mimic or control, and were stained at 48 h without irradiation or 24 h after irradiation. Alexa Fluor 488-conjugated annexin V binding combined with propidium iodide (Molecular Probes, USA) labeling was performed for the distinction of necrotic (annexin V+/propidium iodide+) and apoptotic (annexin V+/propidium iodide−) cells. Apoptotic

### Table 1. Reverse transcription primers for U6 snRNA and miR-148b

| Gene    | Sequence                  |
|---------|---------------------------|
| U6 snRNA| 5'CGCTTCACGAATTTCGCTGCAT3' |
| miR-148b| 5'GTCGTATCAGTGCTGCTGGAGTTGCGACAATTTCGCTGCAT3' |

### Table 2. Quantitative real-time PCR primers for U6 snRNA and miR-148b

| Gene    | Sequence                  |
|---------|---------------------------|
| U6 snRNA| 5'GCTTCGGCAGCACTATACGAAAT3' |
| miR-148b| 5'GGGTCAGTGCTACAGAA3' |

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Fig. 1. Differentially expressed miRNAs in Raji cells after exposure to 2-Gy X-ray compared with unirradiated control cells ($P<0.05$) The irradiated/unirradiated expression ratio was expressed as Log2. The miRPlus™ miRNAs were proprietary miRNAs detected by miRCURY™ LNA Array but not annotated in the miRBase. The bars indicate the means for three independent experiments.
cells were quantified using a BD FACSCanto flow cytometer (BD Biosciences, USA) [21].

Statistical analysis
Each experiment was repeated at least three times. The radiation survival curves were fitted according to the linear-quadratic model: surviving fraction = exp (–αD – βD²). The difference between means was analyzed with the Student’s t-test. All statistical analyses were performed using SPSS (version 17.0) software (SPSS Inc., Chicago, IL, USA). A P value of <0.05 was considered significant.

RESULTS

MiRNA expression profiles
To identify miRNAs implicated in NHL radiosensitivity, miRNA microarray analysis was employed to monitor the expression profiles of miRNAs in Raji cells following radiation treatment. The radiosensitivity of Raji cells was evaluated by clonogenic assay. The radiation dose of 2 Gy, which produced about 50% inhibition of growth and was commonly used as a fractionation dose clinically, was delivered to Raji cells according to the plan described. The miRNA expression was assessed at 4 h post-irradiation by miRNA microarray. MiRNA microarray identified 20 miRNAs as differentially expressed: 10 miRNAs up-regulated and 10 down-regulated (defined as P < 0.05) (Fig. 1).

Quantitative real-time PCR assay
Among the differentially expressed miRNAs, miR-148b was up-regulated 1.53-fold in response to radiation treatment. To confirm the increased expression of miR-148b after irradiation, RNA was extracted from Raji and RL cells at different timepoints post-irradiation of 2 Gy, or at 4 h timepoint post-irradiation of different doses, and analyzed by qRT-PCR. MiR-148b showed an increase at all time and dose points (Fig. 2).

MiR-148b modulated the radiosensitivity of Raji cells
Since miR-148b was up-regulated in response to radiation, we hypothesized that miR-148b could modulate radiation response. Using transient transfection, we up-regulated miR-148b by miR-148b mimic, or down-regulated miR-148b by miR-148b inhibitor in Raji cells. Based on fluorescence microscopy we found that miRNA fragments were successfully transfected into Raji cells with the transfection efficiency >80% (data not shown). Furthermore, the up-regulation and down-regulation of miR-148b expression after transfection was confirmed by qRT-PCR (Fig. 3).

We first evaluated the potential effect of miR-148b on Raji cell growth without irradiation in vitro. The results from cell proliferation assays showed that there was a...
significant decrease in the cell growth rate in miR-148b up-regulated Raji cells compared with the cells transfected with control mimic (Fig. 4A). On the other hand, there was a significant increase in the cell growth rate in miR-148b down-regulated Raji cells (Fig. 4B). Next, we subjected the transfected cells to radiation. A cell proliferation assay showed that after 2- or 4-Gy irradiation, there was increased cell death in both the miR-148b mimic transfected cells and the control compared with non-irradiated groups. However, the miR-148b mimic radiated group exhibited significantly enhanced cell death compared with the control radiated group (Fig. 4C and E). These results support the hypothesis that miR-148b overexpression induces radiation sensitivity of Raji cells. To further confirm the role of miR-148b in radiation sensitivity, we used miR-148b inhibitor to determine whether it could induce radioresistance in Raji cells. As expected, we found that the miR-148b inhibitor transfected groups were significantly more resistant to radiation compared with control groups (Fig. 4D and F). Taken together, these results suggest that

Fig. 4. MiR-148b modulated the proliferation of Raji cells after radiation. Exponentially growing Raji cells were transfected by miR-148b mimic, inhibitor or controls, then treated with different doses of radiation (0, 2, 4 Gy), the cell proliferation was evaluated using a CCK-8 assay. (A, B) Transfected Raji cells received no radiation treatment (0 Gy). (C, D) Transfected Raji cells exposed to 2-Gy X-ray. (E, F) Transfected Raji cells exposed to 4-Gy X-ray. The experiment was performed in triplicate and each value represents the mean ± SE. A paired Student’s t-test indicated statistical significance between the two curves, *P < 0.05.
miR-148b plays an important role in radiation sensitivity of Raji cells.

To further confirm that miR-148b enhances the radiation sensitivity of Raji cells, a clonogenic assay was performed and demonstrated similar effects. Raji cells transfected with the miR-148b mimic had significantly lower colony formation ability after irradiation (Fig. 5A). Conversely, the miR-148b inhibitor-treated Raji cells were significantly more resistant to radiation compared with the control (Fig. 5B). These results provide additional evidence that miR-148b sensitizes Raji cells to radiotherapy.

MiR-148b had no effects on the cell cycle profiles of Raji cells after radiation

Next we determined whether the effect of miR-148b on radiation sensitivity is due to cell cycle alterations. Flow cytometry analysis showed that miR-148b mimic and miR-148b inhibitor had no influence on the cell cycle profiles of Raji cells (Fig. 6A and B). At 24 h after transfection, cells were exposed to X-rays with 0, 2 and 4 Gy, and were analyzed 24 h later. Raji cells showed obvious dose-dependent G2/M arrest after radiation. However, miR-148b mimic and inhibitor had no influence on the cell cycle profiles of post-radiation Raji cells compared with controls (Fig. 6C to F).

MiR-148b enhanced radiation-induced apoptosis of Raji cells

Apoptosis is an important mechanism of cell death in malignancies. To investigate whether miR-148b enhances radiation sensitivity of Raji cells by inducing apoptosis, Raji cells were transfected with miR-148b mimic or control. After incubation for 24 h, these cells were exposed to various doses of irradiation (0, 2 and 4 Gy), and subjected to Annexin V/PI double staining and FACS analysis 24 h later. Untransfected cells were analyzed 24 h after irradiation. As shown in Fig. 7, the percentage of apoptotic cells in the miR-148b overexpression group (miR-148b mimic) was significantly higher than that of the control and untransfected groups at the doses of 0, 2 and 4 Gy (P < 0.05). These data suggest that miR-148b enhances radiation-induced apoptosis of Raji cells.

DISCUSSION

In this study, we employed microarray analysis to examine the miRNA expression profiles of B cell lymphoma cell line Raji cells before and after irradiation. We identified miR-148b as a miRNA that was significantly up-regulated after irradiation in Raji cells, suggesting that it may regulate the radiosensitivity of lymphoma. Functional studies confirmed that miR-148b could inhibit the proliferation and increase the radiosensitivity of Raji cells. Further studies showed that miR-148b increased radiosensitivity probably by promoting radiation-induced apoptosis.

Several studies have suggested that radiation causes altered miRNA expression. In tumor lines including glioblastoma [16, 22], lung cancer [23], colon cancer [24] and prostate cancer [15], radiation noticeably induced changes in the expression of miRNAs. Moreover, the altered miRNAs may be related to cell radiosensitivity. It has been shown that in glioblastoma and prostate cancer cells, radiosensitivity can be modified by altering the expression of specific miRNAs [15, 16]. In addition, recent data showed that radiation changed the levels of miRNA in human endothelial cells which could modulate the intrinsic radiosensitivity of endothelial cells [25]. In fibroblasts, it has been suggested that miRNAs may mediate signaling pathways in response to radiation, and may serve as biodosimetric markers of radiation exposure [26]. These studies indicate...
that altered miRNAs after radiation are involved in the mechanism of radiation response.

Altered miRNAs after radiation may be different due to different radiation doses and modalities. In human B lymphoblastic cell line IM9, different miRNAs were expressed at 1 Gy compared to 10 Gy [27]. In the human lung carcinoma cell line A549, microarray analysis identified 12 and 18 differentially expressed miRNAs in 20- and 40-Gy-exposed A549 cells, respectively [23]. In human colon adenocarcinoma HT-29 cells, different miRNAs may be expressed when radiation was delivered by intensity-modulated radiation therapy compared with conventional radiation therapy [28]. In our study, to explore the role of miRNA in radiosensitivity of NHL, B cell lymphoma cell line Raji was used and received 2 Gy of conventional radiotherapy. Since 2 Gy is the standard fractionation dose and conventional radiotherapy commonly used for NHL at present, the altered miRNAs have apparent clinical significance. After radiation, we identified 10 miRNAs up-regulated and 10 down-regulated, and verified miR-148b as one of the up-regulated miRNAs using quantitative real-time PCR assay.

Fig. 6. miR-148b had no effects on the cell cycle profiles of Raji cells. Data displayed by the DNA content profiles were analyzed, and the cell cycle phase distribution was represented graphically. (A, B) Transfected Raji cells received no radiation treatment. (C–F) At 24 h after transfection, cells transfected with miR-148b mimic (C), control mimic (D), miR-148b inhibitor (E) or control inhibitor (F) received irradiation and were analyzed 24 h later. The experiment was performed in triplicate (P > 0.05).
MiR-148b was selected because its level was significantly changed after radiation. In addition, miR-148b is proposed as a tumor suppressor located at chromosome 12q13 and is down-regulated in oral, pancreatic and colon cancer tissues [29–32]. In gastric and colorectal cancer, miR-148b is frequently down-regulated and acts as a tumor suppressor by inhibiting cell proliferation [32, 33]. However, in esophageal squamous cell carcinoma, miR-148b was up-regulated in the serum and became one of the seven serum miRNA biomarkers of cancer [34].

Few studies address the role of miR-148b in cell radiosensitivity. In this study, miR-148b mimic inhibited the growth of Raji cells, which was consistent with the results in gastric and colorectal cancer [32, 33], and further enhanced the growth inhibition by radiation. Further clonogenic experiments showed that miR-148b mimic could increase the radiosensitivity of Raji cells. In contrast, miR-148b inhibitor could promote the growth of Raji cells and reduced the growth inhibition by radiation. Thus, miR-148b can regulate the proliferation and radiosensitivity of Raji cells.

Cellular response to radiation is related to cell cycle and apoptosis [35]. Therefore, we performed cell cycle and apoptosis assays to explore the mechanism by which miR-148b regulates cell radiosensitivity. The results showed that miR-148b had no apparent influence on the cell cycle profiles of Raji cells. However, the apoptosis assay showed that miR-148b could promote both basic and radiation-induced apoptosis of Raji cells. Collectively, these data suggest that miR-148b increases radiosensitivity through promoting apoptosis.

Radiation-induced apoptosis is considered to be one of the main cell death mechanisms following exposure to irradiation, especially in cells from the lymphoid and myeloid lineages [36]. Cells susceptible to apoptosis more commonly respond to radiation. Radiation-induced cell death is significantly increased in mice mutated for anti-apoptotic members (Bcl-XL, Bcl-2 and Bcl-w) [37, 38]. MiRNAs have recently been shown to regulate apoptosis and may therefore contribute to radiation-induced cell death [39]. In this study, miR-148b was found to promote apoptosis and increase radiation-induced cell death. MiRNAs may target one or simultaneously various mRNAs involved in apoptotic signaling. It has been validated that miR-148 (miR-148a and miR-148b) repressed DNA methyltransferase 3b (DNMT3b) expression [40]. DNMT3b is a major enzyme responsible for the replicating DNA methylation pattern, and gene silencing of both DNMT1 and DNMT3b reduced cell proliferation and sensitized the cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) -mediated cell death [41]. Therefore, we speculate that miR-148b may promote apoptosis and increase radiosensitivity through repressing DNMT3b.

In summary, our results demonstrate that miR-148b plays an important role in the response of NHL to ionizing radiation. MiR-148b could be utilized to predict the...
radiosensitivity of NHL. In non-small cell lung cancer patients, miRNAs were differentially expressed after postoperative radiotherapy and related to the overall survival or local and distant recurrence rates [42]. In addition, modulation of miR-148b expression may be a promising treatment option. However, since the present study focused mainly on one NHL cell line, the conclusion should be verified in other NHL cell lines, animal models and human. Further studies will promote our deeper understanding of the regulation mechanism of miR-148b as a radiation sensitizer.

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