MiRNA-203 Reduces Nasopharyngeal Carcinoma Radioresistance by Targeting IL8/AKT Signaling

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Abstract

Radioresistance poses a major challenge in nasopharyngeal carcinoma (NPC) treatment, but little is known about how miRNA (miR) regulates this phenomenon. In this study, we investigated the function and mechanism of miR-203 in NPC radioresistance, one of downregulated miRs in the radioresistant NPC cells identified by our previous microarray analysis. We observed that miR-203 was frequently downregulated in the radioresistant NPC tissues compared with radiosensitive NPC tissues, and its decrement significantly correlated with NPC radiosensitivity and poor patient survival, and was an independent predictor for reduced patient survival. In vitro radioresistance assays showed that miR-203 mimic markedly decreased NPC cell radioresistance. In a mouse model, therapeutic administration of miR-203 agomir dramatically sensitized NPC xenografts to irradiation. Mechanistically, we confirmed that IL8 was a direct target of miR-203, and found that reduced miR-203 promoted NPC cell radiosensitivity by activating IL8/AKT signaling. Moreover, the levels of IL8 and phospho-AKT were significantly increased in the radioresistant NPC tissues compared with radiosensitive NPC tissues, and negatively associated with miR-203 level. Our data demonstrate that miR-203 is a critical determinant of NPC radioresponse, and its decrement enhances NPC radioresistance through targeting IL8/AKT signaling, highlighting the therapeutic potential of the miR-203/IL8/AKT signaling axis in NPC radiosensitization.

Introduction

Nasopharyngeal carcinoma (NPC) is the most frequent head and neck tumor in southern China and Southeast Asia, which poses one of the most serious public health problems in these areas (1). Radiotherapy is the major therapeutic modality used to treat NPC. Although NPC is sensitive to radiotherapy, a major impediment to achieve long-term survival is radioresistance that has been linked to an increased likelihood of recurrence and a distant metastasis (2, 3).

MiRNAs (miR) are endogenous small non-coding RNAs that act as crucial gene regulators at posttranscriptional level by binding to the 3’ untranslated region (UTR) of target mRNAs. MiRs are believed to have fundamental roles in the human cancers, and have great potential in the diagnosis and treatment of cancers (4). Regulation of tumor radiosensitivity via miRs-associated mechanisms has attracted much attention in the recent years (5–8). Over the past few years, several miRs involving in tumor radioresistance such as miR-21 (9), miR-95 (10), miR-23b (11), let7 (12), miR-205 (13), miR-210 (14), miR-181a (15), miR-125b (16), and miR-324-3p (17) have been identified.

We previously used microarrays to compare the differences of miR and mRNA expression profiles in the NPC cell lines with different radiosensitivities, and found that miR-203 is downregulated, but IL8 is upregulated in the radioresistant NPC cells (18). Bioinformatics analysis identified IL8 as a putative target gene of miR-203 (18). MiR-203 is frequently downregulated and functions as a potential tumor suppressor in the various types of cancers, including NPC (19–21). Recent studies showed that the miR-203 expression level positively correlates with chemosensitivity in colon (22) and prostate (23) cancer cells. However, the function and mechanism of miR-203 in tumor radiosensitization have not been characterized.

IL8 is a proinflammatory cytokine-X-cysteine (CXC) chemokine (24). Previous studies have shown that IL8 promotes tumor angiogenesis, growth, and metastasis in the various types of cancers, including NPC (25–31). Increased serum and tissue IL8 levels are associated with worse prognosis of NPC patients, and can serve as an independent prognostic factor for overall patient survival (31, 32). However, it is undetermined whether high IL8 expression in NPC contributes to radioresistance, leading to worse patient prognosis. IL8 executes its biologic functions by activating cellular signaling pathways. AKT signaling plays a role in the tumorigenesis and progression of NPC (33). It has been reported that irradiation of NPC cells can activate AKT (34), and activated AKT is associated with NPC radioresistance (13, 35). Activation of AKT by IL8 signaling, which promotes tumor metastasis, has been shown in NPC (31). However, it is unknown whether activation of AKT by IL8 increases NPC radioresistance.
In this study, we found that miR-203 expression was frequently downregulated in the radioresistant NPC tissues, restoration of miR-203 expression decreased NPC radioresistance both in vitro and in vivo, and reduced miR-203 increased NPC radioresistance by targeting IL8/AKT signaling. Our data strongly suggest that the miR-203 expression level could serve as a potential marker for predicting NPC radioresponse and patient prognosis, and NPC patients with reduced miR-203 may benefit from specific targeted therapies directed at miR-203/IL8/AKT signaling.

Materials and Methods

Cell lines
Radioresistant human NPC cell lines CNE2-IR and CNE1-IR cells as well as their corresponding radiosensitive cell lines CNE2 and CNE1 cells were previously established by us (36, 37), and cultured with RPMI-1640 medium containing 10% FBS (Invitrogen). Radioresistant CNE2-IR and CNE1-IR cells were derived from parental CNE2 and CNE1 cells, respectively, by treating the cells with four rounds of sublethal ionizing radiation (36, 37). Radiosensitive CNE2 and CNE1 cells, used as a control, were treated with the same procedure except sham irradiated. Experiments were performed with the CNE2-IR and CNE1-IR cells within 4 to 10 passages after the termination of irradiation, and their radioresistance was tested by a clonogenic survival assay before use.

Patients and tissue samples
One hundred and eleven NPC patients without distant metastasis (M0 stage) at the time of diagnosis who were treated by radical radiotherapy alone in the Affiliated Cancer Hospital of Central South University, China between January 2006 and December 2008 were recruited in this study. The radiotherapy was administered for a total dose of 60–70 Gy (2 Gy/fraction, 5 days a week). The neck received 60 Gy for lymph node–negative cases and 70 Gy for lymph node–positive cases. NPC tissue biopsies were obtained at the time of diagnosis before any therapy, fixed in 4% formalin and embedded in paraffin. We also acquired 16 cases of formalin-fixed and paraffin-embedded normal nasopharyngeal mucosa in the same period. On the basis of the 1978 WHO classification (38), all tumors were histopathologically diagnosed as poorly differentiated squamous cell carcinomas (WHO type III). The clinical stage of the patients was classified according to the 2008 NPC staging system of China (39).

The radiotherapy response was evaluated clinically for primary lesions based on nasopharyngeal fiberscope and MRI one month after the initiation of radiotherapy according to the following criteria. Radioresistant NPC patients were defined as ones with persistent disease (incomplete regression of primary tumor and/or neck lymph nodes) at >3 months or with local recurrent disease at the nasopharynx and/or neck lymph nodes at ≤12 months after completion of radiotherapy. Radiosensitive NPC patients were defined as ones without local recurrent lesions (complete regression) at >3 months and without local recurrent disease at >12 months after completion of radiotherapy. On the basis of the above criteria, 111 NPC patients comprised 53 radioresistant and 58 radiosensitive ones.

The patients were followed up, and the follow-up period at the time of analysis was more than 72 months (average, 77.5 ± 11.8 months). Disease-free survival (DFS) was calculated as the time from the completion of primary radiotherapy to the date of pathologic diagnosis or clinical evidence of local failure and/or distant metastasis. Overall survival (OS) was defined as the time from the initiation of primary radiotherapy to the date of cancer-related death or when censored at the latest date if patients were still alive. The clinicopathologic parameters of the patients used in the present study are shown in Supplementary Table S1.

RNA quantification
Total RNA was extracted from NPC cells with TRizol reagent (Invitrogen), or from paraffin-embedded NPC tissues with the RecoverAll Total Nucleic Acid Isolation Kit (Ambion) according to the manufacturer’s instructions. qRT-PCR detection of miR-203 and IL8 expression in NPC cells and tissues was performed as previously described (18). The products were quantitated using the 2^(-ΔΔCt) method against GAPDH or U6 for normalization. The primer sequences are listed in Supplementary Table S2.

3’UTR dual luciferase reporter assay
A dual luciferase reporter plasmid with IL8 3’UTR (HmiT009678-MT01; GeneCopoeia), without IL8 3’UTR of (CmiT000001-MT01; GeneCopoeia), or with mutated IL8 3’UTR in the predicted miR-203–binding sites constructed by GeneCopoeia, and miR-203 or control mimic (Ribobio) were cotransfected into NPC cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were harvested 48 hours after transfection, both firefly luciferase and renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega) according to the manufacturer’s instruction, and luciferase activity was estimated using a luminometer (Promega).

Transfection of miR-203 mimic and inhibitor into NPC cells
Cells were cultured with RPMI-1640 medium containing 10% FBS overnight, and then 50 μmol/L miR-203 mimic, miR-203 inhibitor, and their respective negative control (Ribobio) were transfected into cells using the riboFect CP Transfection Kit (Ribobio) according to the manufacturer’s instruction, respectively. Twenty-four hours after transfection, cells were subjected to further analysis.

Generation of NPC cell lines with stable knockdown of IL8
Psi-LVR16GP-IL8 shRNA and psi-LVR16GP-scramble non-target shRNA vectors, which were established by GeneCopoeia and confirmed by sequencing, were transfected into CNE2-IR cells using Lipofectamine 2000, respectively. Cells were selected using puromycin for 2 weeks, and stable knockdown of IL8 CNE2-IR cell lines and control cell lines were obtained. The targets for human IL8 shRNAs are shown in Supplementary Fig. S1.

Clonogenic survival assay
A clonogenic survival assay was performed as previously described by us (36). Briefly, cells were exposed to a range of radiation doses (1–8 Gy) and 12 days after irradiation surviving colonies were stained with 0.5% crystal violet and counted. The survival fraction was calculated as the numbers of colonies divided by the numbers of cells seeded times plating efficiency. Radiation dose–response curves were created by fitting the data to the linear quadratic equation $S = e^{-\alpha D - \beta D^2}$ using GraphPad Prism 5.0, where S is the surviving fraction, $\alpha$ and $\beta$ are inactivation
constants, and D is the dose in Gy. The ALIC that represent the mean inactivation dose (MID) was calculated using GraphPad Prism 5.0. The radiation protection factor (RPF) was calculated by dividing the MID of the test cells by the MID of control cells.

In vivo tumor radioreponse assay
Nude male mice that were 4-weeks-old were obtained from the Laboratory Animal Center of Central South University (Changsha, China). A total of 5 × 10⁶ CNE2-IR cells were injected s.c. into the right flanks of 5-week-old nude mice. When the xenograft volumes reached approximately 50 mm³, the mice were randomly divided into 2 groups (n = 5 mice each), 5 nmol control or miR-203 agomir (Ribo-Bio) in 25 μL saline buffer was intratumorally injected into the tumor mass at multiple sites per mouse, and next day an 8-Gy dose of ionizing radiation was delivered to the tumor. Three days after irradiation, 5 nmol control or miR-203 agomir was intratumorally injected into the tumor mass at multiple sites per mouse. Five weeks after irradiation, the mice were killed by cervical dislocation, and their tumors were excised, weighted, and cut in half, with one half fixed in formalin for TUNEL and immunohistochemical staining, and the remaining half flash-frozen in liquid nitrogen until use. Tumor volume (in mm³) was measured by caliper measurements performed every 3 to 4 days and calculated by using the modified ellipse formula (volume = length × width²/2).

Cell-cycle analysis
Flow-cytometry analysis of cell cycle in response to irradiation was performed as previously described by us (36).

Hoechst 33258 staining
Hoechst 33258 staining was performed to detect apoptotic cells after irradiation as previously described by us (37).

Western blotting
Proteins were extracted from cells and tissues. An equal amount of protein in each sample was subjected to SDS-PAGE separation, followed by blotting onto a polyvinylidene difluoride membrane. After blocking, blots were incubated with anti-IL8 (ab18672; Abcom), phospho-AKT (Thr308; #4056; Cell Signaling Technology), or AKT antibody (#4691; Cell Signaling Technology) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The signal was visualized with an enhanced chemiluminescence detection reagent (Pierce). β-Actin was detected as a loading control.

In situ detection of apoptotic cells
Terminal deoxynucleotidyl transferase(TdT)–mediated dUTP nick end labeling (TUNEL) was performed to detect apoptotic cells of formalin-fixed and paraffin-embedded tissue sections of xenograft tumors after irradiation with the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s instruction. Quantitative evaluation of apoptotic cells was done by examining the sections in 10 random microscopic fields and counting the number of TUNEL-positive cancer cells among 1,000 carcinoma cells under the light microscope. The apoptotic index was expressed as positive cells per 100 cancer cells.

Immunohistochemistry
Immunohistochemical staining of IL8, phospho-AKT (Thr308), and γH2AX (phospho-S139) was performed on formalin-fixed and paraffin-embedded tissue sections. Brieﬂy, after antigen retrieval tissue sections were incubated with anti-IL8 antibody (ab18672; Abcom), phospho-AKT1 (Thr308; ab5626; Abcom), or γH2AX antibody (ab2893; Abcom) overnight at 4°C, and then were incubated with biotinylated secondary antibody followed by avidin–biotin peroxidase complex (DAKO). Finally, tissue sections were incubated with 3’, 3’-diaminobenzidine (Sigma) and counterstained with hematoxylin. In negative controls, primary antibodies were omitted. The immunoreactions were evaluated independently by two pathologists as described previously. Staining intensity was categorized: Absent staining as 0, weak as 1, moderate as 2, and strong as 3. The percentage of stained cells was categorized as no staining = 0, <30% of stained cells = 1, 30% to 60% = 2, and >60% = 3. The staining score (ranging from 0 to 6) for each tissue was calculated by adding the area score and the intensity score. A combined staining score of ≤3 was considered to be low expression, and a score of >3 was considered to be high expression.

Statistical analysis
All experiments were carried out at least three times. Data were presented as the mean ± SD. Statistical analysis was conducted using SPSS 20.0 software. For comparisons between two groups, a Student t test or χ² test was used. Survival curves were obtained by

ELISA
Cells were cultured with RPMI-1640 medium containing 10% FBS for 24 hours, and then incubated in a serum-free medium for an additional 12 hours. Conditioned medium was collected, followed by filtration to remove the debris, and IL8 concentration in the conditioned medium was measured using the Human IL8 Sandwich ELISA Kit (Excell) according to the manufacturer’s instruction.

Antibody neutralization of secretory IL8
Cells were cultured with RPMI-1640 medium containing 2% FCS and 2.5 μg/ml mouse anti-human IL8 antibody (ab18672; Abcom) or 2.5 μg/ml mouse control IgG1 (ab188776; Abcom) for 24 hours, and then cells were subjected to further analysis.

Stimulation of cells by IL8
Cells were cultured with RPMI-1640 medium containing 10% FBS for 24 hours, and incubated in a serum-free medium for an additional 12 hours, and then recombinant human IL8 (Life Technologies) were added into the medium. Twelve hours after stimulation, cells were subjected to further analysis.

Transfection of AKT1 DNaZyme
Cells were cultured with RPMI-1640 medium containing 10% FBS one day before transfection. The transfection of 2 μmol/L DNaZyme-targeting AKT1 (the sequence: 5’TGGTCCACAGGC-TAGCTACAACGACCTCAGCCAGC-3’) was performed with oligo-lectamine reagent (Life technologies) as previously described (40). Forty-eight hours after transfection, cells were subjected to further analysis.

Statistical analysis
All experiments were carried out at least three times. Data were presented as the mean ± SD. Statistical analysis was conducted using SPSS 20.0 software. For comparisons between two groups, a Student t test or χ² test was used. Survival curves were obtained by
using the Kaplan–Meier method, and comparisons were made by using the log-rank test. Univariate and multivariate survival analyses were conducted on all parameters by using the Cox proportional hazards regression model. The Spearman rank correlation coefficient was used to determine the correlation between two parameters. *P* values less than 0.05 were considered to be statistically significant.

Ethics statement
This study was approved by the ethics committee of Xiangya School of Medicine, Central South University, China. Written informed consent was obtained from all participants in the study. All animal experiments were undertaken in accordance with the Guide for the Care and Use of Laboratory Animals of Central South University, with the approval of the Scientific Investigation Board of Central South University.

Results
Reduced miR-203 expression is correlated with NPC radioresistance and poor patient prognosis
Our previous integrated analysis of differential miR and mRNA expression profiles in the radioresistant CNE2-IR and radiosensitive CNE2 cells identified 11 differential miRs anti-correlated with mRNA expression (Supplementary Table S3; ref. 18), including miR-23a and let-7e, whose family members have been demonstrated to be associated with tumor radiosresponse (11, 12, 41). We were interested in reduced miR-203 in the CNE2-IR cells, because the function and mechanism of which in tumor radioresistance have not been characterized.

Using qRT-PCR, we confirmed that miR-203 expression was significantly decreased in the CNE2-IR cells compared with CNE2 cells, and in the additional radioresistant NPC CNE1-IR cells compared with radiosensitive CNE1 cells (Fig. 1A). We proceeded to detect miR-203 levels in a cohort of NPC tissues. Compared with the radiosensitive NPCs, miR-203 expression was significantly decreased in the radioresistant NPCs (Fig. 1A), and negatively correlated with NPC radioresistance (*r* = -0.70, *P* < 0.001). The cutoff value of miR-203, which could be used to differentiate between the radioresistant and radiosensitive NPCs, was 1.05. It was determined by receiver-operating characteristic analysis (Supplementary Fig. S2) and used to separate the patients into low and high miR-203 level groups. Survival analyses revealed that low miR-203 level in NPCs correlated with the markedly reduced DFS and OS of the patients (Fig. 1B). A univariate Cox regression analysis showed that the miR-203 expression level and clinical TNM stage significantly affected the DFS and OS of NPC patients (Table 1). A multivariate Cox regression analysis confirmed that low miR-203 expression was an independent predictor for the reduced DFS and OS of NPC patients (Table 1). These results indicated the importance of the miR-203 expression level in the NPC radioresistance and patient prognosis.

MiR-203 decreases NPC cell radioresistance in vitro
To determine the effect of reduced miR-203 on NPC cell radioresistance in vitro, CNE2-IR cells were transiently transfected with control or miR-203 mimic, and then cell radiosensitivity was determined. A clonogenic survival assay showed that transfection...
of miR-203 significantly decreased CNE2-IR cell radioresistance compared with transfection of control mimic [AUC, 1.14 (miR-203 mimic) vs. 1.86 (control mimic); \( P < 0.05; \text{RPF} = 0.61; \) Fig. 2A]. Irradiation primarily leads to double-strand DNA breaks (DSB), and unrepaird or misrepaired DSBs in the DNA lead to cell apoptosis. The apoptosis resulting from irradiation is, to a considerable degree, understood as radiosensitivity (41). Therefore, we also analyzed the effect of miR-203 mimic on the irradiation-induced apoptosis of CNE2-IR cells. Hoechst 33258 staining showed that transfection of miR-203 mimic significantly increased irradiation-induced apoptosis of CNE2-IR cells compared with transfection of control mimic (Fig. 2B). Our previous study showed that compared with radiosensitive CNE-2 cells, more CNE-2-IR cells were found detained in S phase with less cells in G2 phase at 24 hours after 6 Gy irradiation, whereas compared with control mimic–transfected cells, less miR-203 mimic–transfected cells were found detained in S phase with more miR-203 mimic–transfected cells in G2–M phase, which is consistent with the typical radiosensitive phenotype (42). Accordingly, the difference in response to irradiation between control or miR-203 mimic–transfected CNE2-IR cells was further studied by cell-cycle analysis using flow cytometry. As showed in Fig. 2C, no difference was induced by irradiation in G0–G1 phase at 24 hours after 6 Gy irradiation, whereas compared with control mimic–transfected cells, less miR-203 mimic–transfected cells were found detained in S phase with more miR-203 mimic–transfected cells in G2–M phase, which is consistent with the typical radiosensitive phenotype (42). Moreover, transfection of miR-203 mimic into the additional radioresistant NPC CNE1-IR cells was also able to reduce cell radioresistance [AUC, 1.97 (miR-203 mimic) vs. 2.56 (control mimic); \( P < 0.05; \text{RPF} = 0.77; \) Fig. 2A]. Taken together, these results demonstrated that restoration of miR-203 expression in the radioresistant NPC cells significantly decreased in vitro cell radioresistance, i.e. that miR-203 could sensitize NPC cells to irradiation.

**MiR-203 decreases NPC cell radioresistance in vivo**

To determine the effect of reduced miR-203 on NPC radioresponse in vivo, we generated subcutaneous tumors in nude mice using CNE2-IR cells. Control or miR-203 agomir was injected into the tumors before and after 8 Gy ionizing radiation, and then tumor radioresponse was assessed. As shown in Fig. 3A, radioresistance of miR-203 agomir–injected tumors was significantly lower than that of control agomir–injected tumors as demonstrated by tumor growth and weight. Hematoxylin and eosin (H&E) staining of tumor tissue sections showed that more necrosis was noted in miR-203 agomir–injected tumors compared with control agomir–injected tumors [29.90% ± 4.16% (miR-203 agomir) vs. 13.32% ± 3.53% (control agomir); \( P < 0.01; \) Fig. 3B]. TUNEL assay showed that more apoptotic cells were present in the miR-203 agomir–injected tumors relative to control agomir–injected tumors [27.3% ± 4.21% (miR-203 agomir) vs. 8.92% ± 1.60% (control agomir); \( P < 0.01; \) Fig. 3C]. Immunohistochemical staining indicated that more positive cells of γH2AX, that is, more cells with DNA damage, were present in the miR-203 agomir–injected tumors compared with control agomir–injected tumors [18.35% ± 4.28% (miR-203 agomir) vs. 7.34% ± 2.27% (control agomir); \( P < 0.01; \) Fig. 3D]. Taken together, these results demonstrated that restoration of miR-203 expression obviously decreased in vivo NPC radioresistance, suggesting that in vivo administration of miR-203 agomir had a considerable potential for NPC radiosensitization.

**MiR-203 regulates NPC cell radioresponse through targeting IL8**

To confirm IL8 as a direct target of miR-203, we cotransfected a dual luciferase reporter plasmid with wild-type (wt) IL8 3′UTR into CNE2-IR cells with control or miR-203 mimic. The results revealed a significant reduction in luciferase activity in miR-203 mimic–transfected cells compared with control mimic–transfected cells, whereas miR-203 mimic had no obvious effects on the luciferase activity of a dual luciferase reporter plasmid without IL8 3′UTR or with mutated IL8 3′UTR in the two miR-203–binding sites (Fig. 4A). Furthermore, cellular and secretory IL8 levels were significantly decreased in the miR-203 mimic–transfected CNE2-IR cells, whereas significantly increased in the

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**Table 1.** Univariate and multivariate analyses of prognostic factors for OS and DFS using the Cox proportional hazards regression model (\( N = 111 \))

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>DFS Multivariate analysis</th>
<th>OS Multivariate analysis</th>
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<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
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<td>≥46</td>
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<td>1.000</td>
<td>1.000</td>
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<td>&lt;46</td>
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<tr>
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<td>Lymph node (N) metastasis</td>
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<td>1.000</td>
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<td>0.259a (0.304–0.875)</td>
<td>0.358 (0.193–0.664)</td>
</tr>
</tbody>
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Abbreviation: CI, confidence interval; HR, hazard ratio.

\(* P < 0.05.\)

\( P < 0.01.\)

\( P < 0.001.\)
miR-203 inhibitor–transfected CNE2 cells compared with their corresponding controls (Fig. 4B). Taken together, these results proved that IL8 was a direct target of miR-203 in NPC cells. Notably, IL8 level was significantly downregulated in the miR-203 agomir–injected xenografts compared with control agomir–injected xenografts (Fig. 4C), supporting our in vitro results.

Figure 2.
Upregulation of miR-203 decreases NPC cell radioresistance in vitro. A, a clonogenic survival assay shows that transfection of miR-203 mimic decreased NPC cell radioresistance compared with transfection of control mimic. Left, CNE2-IR and CNE1-IR cells transiently transfected with control or miR-203 mimic were irradiated with a range of 1 to 8 Gy radiation doses, and colonies that formed after incubation of 12 days were stained with crystal violet and photographed; right, dose survival curves were created by fitting surviving fractions to the linear quadratic equation. B, Hoechst 33258 staining shows that transfection of miR-203 mimic increased the irradiation-induced apoptosis of CNE2-IR cells compared with transfection of control mimic. Left, CNE2-IR and CNE2-IR cells transiently transfected with control or miR-203 mimic were exposed to 6 Gy ionizing radiation, incubated for 72 hours, and stained with Hoechst 33258 and photographed; right, a histogram shows the apoptotic rate of CNE2-IR cells and its transfectants. C, a flow-cytometry analysis of cell cycle shows that miR-203 mimic–transfected CNE2-IR cells were blocked at G2-M phase by irradiation. Left, a representative result of cell-cycle distribution of control or miR-203 mimic–transfected CNE2-IR cells at 24 hours after 6 Gy irradiation. Right, a histogram shows percentages of cells at each cycle phase in the control or miR-203 mimic–transfected CNE2-IR cells. Three experiments were done; means, SDs, and statistical significance are denoted; *, P < 0.05; **, P < 0.01; ns, nonsignificant difference.
Figure 3. Upregulation of miR-203 decreases NPC cell radiosensitivity in vivo. A, the growth and weight of control or miR-203 agomir–injected CNE2-IR xenograft tumors after irradiation. Left, 5 nmol control or miR-203 agomir was injected into CNE2-IR xenografts before and after 8 Gy ionizing radiation. Five weeks after irradiation, the mice were killed, and the tumors were photographed; middle, the growth curves of control or miR-203 agomir–injected CNE2-IR tumors (n = 5 each group) at sacrifice with respect to the first measurements after irradiation; right, the average weights of control or miR-203 agomir–injected CNE2-IR tumors (n = 5 each group) at sacrifice. B, left, a representative image of H&E staining of control or miR-203 agomir–injected CNE2-IR xenografts with regions of necrosis outlined after irradiation; right, a histogram shows percentages of necrosis areas in the tumors (n = 5 each group). C, left, a representative image of TUNEL detection of apoptotic cells in the control or miR-203 agomir–injected CNE2-IR xenografts after irradiation; right, a histogram shows percentages of apoptotic cells in the tumors (n = 5 each group). D, left, a representative image of immunohistochimerical staining for γH2AX in the control or miR-203 agomir–injected CNE2-IR xenografts after irradiation; right, a histogram shows percentages of γH2AX-positive cells in the tumors (n = 5 each group). Means, SDs, and statistical significance are denoted: *, P < 0.05; **, P < 0.01. Original magnification, ×200.

Next, we investigated whether IL8 mediates miR-203–regulated NPC radiosensitivity. CNE2-IR cell lines with stable knockdown of IL8 and control cell lines were established (Fig. 5A and Supplementary Fig. S1). A clone survival assay showed that IL8 knockdown decreased cell radiosensitivity [AUC, 1.03 (IL8 shRNA 1) vs. 1.72 (scramble shRNA), P < 0.05; RPF = 0.58; AUC, 0.98 (IL8 shRNA 2) vs. 1.72 (scramble shRNA), P < 0.05; RPF = 0.55; Fig. 5B], phenocopying that seen in the miR-203 mimic–transfected CNE2-IR cells. Moreover, neutralization of secretory IL8 using anti-IL8 antibody decreased CNE2-IR cell radiosensitivity compared with control IgG [AUC, 1.42 (IL8 antibody) vs. 1.74 (control IgG), P < 0.05; RPF = 0.81; Fig. 5C]. We also investigated whether exogenous IL8 increases NPC cell radiosensitivity, and found that exogenous IL8 stimulation increased radiosensitivity of radiosensitive CNE-2 cells as demonstrated by a clone survival assay [AUC, 1.28 (1.5 ng/ml IL8) vs. 1.01 (control), P < 0.05; RPF = 1.27; AUC, 1.48 (4.5 ng/ml IL8) vs. 1.01 (control), P < 0.05; RPF = 1.46; Fig. 5D]. Importantly, exogenous IL8 stimulation markedly abolished the radiosensitizing effect of miR-203 mimic in the radiosensitive CNE2-IR cells [AUC, 1.59 (miR-203 mimic plus IL8) vs. 1.12 (miR-203 mimic), P < 0.05; RPF = 1.42; Fig. 5E], and IL8 knockdown markedly abolished radiosensitivity induced by transfection of miR-203 inhibitor in the radiosensitive CNE2 cells [AUC, 1.04 (miR-203 inhibitor plus IL8 knockdown) vs. 1.43 (miR-203 inhibitor plus scramble shRNA), P < 0.05; RPF = 0.73; Fig. 5F]. Taken together, our results demonstrated that miR-203 regulated NPC cell radiosensitivity through targeting IL8, and reduced miR-203 increased NPC radiosensitivity by upregulating IL8.

IL8/AKT signaling mediates miR-203–regulated NPC cell radiosresponse

Previous studies indicated that activated AKT plays a critical role in NPC radiosensitivity (13, 34, 35), and IL8 can activate AKT in NPC (31). Therefore, we investigated whether IL8/AKT signaling mediates miR-203–regulated NPC cell radiosresponse. Western blotting showed that either IL8 stimulation or transfection of miR-203 inhibitor significantly enhanced phospho-AKT level in the radiosensitive CNE2 cells, whereas either IL8 knockdown or transfection of miR-203 mimic significantly reduced phospho-AKT level in the radiosensitive CNE2-IR cells (Fig. 6A). Importantly, IL8 knockdown abrogated increment of phospho-AKT level induced by transfection of miR-203 inhibitor in the radiosensitive CNE2 cells, and IL8 stimulation restored phospho-AKT...
level decreased by transfection of miR-203 mimic in the radioresistant CNE2-IR cells (Fig. 6A). Moreover, a clone survival assay showed that either transfection of AKT DNAzyme or PI3 kinase inhibitor LY294002 treatment decreased CNE2-IR cell radioresistance [AUC, 1.32 (DNAzyme) vs. 1.82 (vehicle), P \textless 0.05; RPF = 0.73; AUC, 1.31 (LY294002) vs. 1.83 (vehicle), P \textless 0.05; RPF = 0.72; Fig. 6B]. Importantly, both transfection of AKT DNAzyme and LY294002 treatment significantly abolished radioresistance induced by IL8 stimulation in the radiosensitive CNE2 cells [AUC, 1.11 (IL8 plus LY294002) vs. 1.51 (IL8), P \textless 0.05; RPF = 0.73; AUC, 1.17 (IL8 plus DNAzyme) vs. 1.51 (IL8), P \textless 0.05; RPF = 0.77; Fig. 6C].

In the cohort of NPC tissues, the levels of IL8 and phospho-AKT were significantly higher in the radioresistant NPCCs than those in the radiosensitive NPCCs (Fig. 6D; Table 2). Correlation analyses revealed that IL8 level was positively associated with phospho-AKT level (r = 0.57, P < 0.001), whereas negatively associated with miR-203 level (r = −0.70, P < 0.001), and phospho-AKT level was negatively associated with miR-203 level (r = −0.61, P < 0.001; Supplementary Fig. S3). Moreover, phospho-AKT expression was significantly reduced in the miR-203 agomir–injected xenografts relative to control agomir–injected xenografts (Fig. 4C). Taken together, these results suggested that IL8/AKT signaling mediated miR-203–regulated NPC cell radioresponse, and reduced miR-203 increased NPC radioresistance by activating IL8/AKT signaling.

**Discussion**

Radioresistance is the main obstacle in the clinical management of NPC (2, 3). Investigating the role of miRs in radioresistance is a promising avenue, given their ability to regulate multiple oncogenic processes, including response to therapy (8). In this study, we focused on miR-203, one of downregulated miRs in the radioresistant NPC cells (18), because the roles of miR-203 in tumor radioresistance are unknown.

To gain insight into miR-203 function in NPC radioresistance, we performed in vitro and in vivo radioresponse assays, and found that upregulation of miR-203 decreased radioresistance in NPC cells and xenograft tumors, demonstrating that reduced miR-203 enhances NPC radioresistance. These findings are clinically relevant, given our discovery that miR-203 was frequently downregulated in the radioresistant NPC tissues, and its decrement correlated with NPC radioresistance and poor patient survival, outlining a potential marker for predicting the radioresponse and prognosis of NPC patients. To our
knowledge, it is for the first time reported that miR-203 modulates tumor radioresponse.

Our previous bioinformatics analysis identified IL8 as one of miR-203 potential target genes (18). In this study, we proved that IL8 is a direct target of miR-203 in NPC cells, which is for first time reported IL8 as a miR-203 target in tumor cells. Next, we investigated whether IL8 mediates miR-203–regulated NPC radioresponse. We observed that IL8 knockdown or antibody
neutralization of secretory IL8 decreased NPC cell radioresistance, whereas IL8 stimulation increased NPC cell radioresistance. Furthermore, IL8 stimulation abolished the radiosensitizing effect of miR-203 mimic, and IL8 knockdown abolished radioresistance induced by transfection of miR-203 inhibitor in NPC cells. In the clinical NPC samples, IL8 level was significantly higher in the radioresistant NPCs than that in the radiosensitive NPCs, and negatively associated with miR-203 level. These results demonstrate that reduced miR-203 enhances NPC radioresistance through targeting IL8.

miRs modulate tumor radiosensitivity by targeting signal pathways (8). Previous studies indicated that activated AKT is associated with NPC radioresistance (13, 34, 35) and IL8 can activate AKT in NPC (31). Therefore, we investigated whether IL8 mediates miR-203–regulated NPC cell radioresponse by activating AKT. We observed that transfection of miR-203 inhibitor or IL8 stimulation enhanced, whereas transfection of miR-203 mimic or IL8 knockdown reduced phospho-AKT level in NPC cells. Importantly, IL8 knockdown abrogated increment of phospho-AKT level induced by transfection of miR-203 inhibitor, and IL8 stimulation restored phospho-AKT level decreased by transfection of miR-203 mimic in NPC cells. miR-203 agomir reduced the levels of IL8 and phospho-AKT in the NPC cell xenografts. Moreover, AKT

Table 2. The levels of IL8 and phospho-AKT in the normal nasopharyngeal mucosa and nasopharyngeal carcinoma tissues

<table>
<thead>
<tr>
<th></th>
<th>NNMT</th>
<th>Radiosensitive NPC</th>
<th>Radioresistant NPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (0–3)</td>
<td>16</td>
<td>40</td>
<td>11</td>
</tr>
<tr>
<td>High (4–6)</td>
<td>0</td>
<td>18</td>
<td>42</td>
</tr>
<tr>
<td>Phospho-AKT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (0–3)</td>
<td>16</td>
<td>38</td>
<td>16</td>
</tr>
<tr>
<td>High (4–6)</td>
<td>0</td>
<td>20</td>
<td>37</td>
</tr>
</tbody>
</table>

NOTE: P < 0.01 by the z2 test. NNMT vs. radiosensitive NPC; NNMT vs. radioresistant NPC; radiosensitive NPC vs. radioresistant NPC. Abbreviation: NNMT, normal nasopharyngeal mucosal tissue.
DNAzyme or LY294002 not only decreased NPC cell radioresistance, but also markedly abrogated NPC cell radioresistance induced by IL8 stimulation. In the clinical NPC samples, phospho-AKT level was significantly higher in the radioresistant NPC tissues than that in the radiosensitive NPC tissues, and positively associated with IL8 level whereas negatively associated with miR-203 level. Taken together, our results demonstrate that activation of AKT by IL8 enhances NPC radioresistance, and suggest that reduced miR-203 increases NPC radioresistance by activating IL8/AKT signaling.

Methods for radiosensitization of NPC attract much attention (43–45). In this study, we confirmed that restoration of miR-203 expression by using miR-203 agomir enhanced NPC radiosensitivity in NPC xenografts, suggesting its considerable potential in radiosensitizing NPC. Nucleic-acid drugs, such as miRs, can be directly synthesized and modified to be more lipophilic that improves penetration. Such modification includes cholesterylation. Our delivery of miR-203 agomir, cholesterylated miR mimic, successfully increased NPC radiosensitivity in the intratumoral injection model, suggesting that miR-203 agomir has a potential for further drug development. Our results also highlight the possibility of radiosensitizing NPC with reduced miR-203 by inhibiting IL8/AKT signaling, given that increased level of IL8 and phospho-AKT was seen in the radiosensitive NPC tissues, and AKT DNAzyme or LY294002 significantly abrogated NPC cell radioresistance.

Although IL8/AKT signaling seems to largely account for the radioresistant phenotype of NPC cells induced by reduced miR-203, indeed a single miR has been thought to target multiple miRNAs to regulate gene expression (46). Therefore, there may be other molecules or signaling pathways that are also targeted by miR-203 (19, 22, 47–50), and some of them may be still unknown in NPC. This presumption may raise interesting future work to reveal the entire functions of miR-203 in NPC radioresistance. Moreover, our results indicated that miR-203 is a potential marker for predicting the radiorepro and prognosis of NPC patients, but this study was a retrospective study that is limited to only 111 patients from a single institution. Therefore, a prospective study involving larger populations from multiple centers will be required to further validate the usefulness of miR-203.

In summary, our data demonstrate that (i) miR-203 is frequently downregulated in the radioresistant NPC tissues, and its decrement significantly correlates with NPC radioresistance, and is an independent predictor for the poor survival of NPC patients; (ii) upregulation of miR-203 decreases NPC cell radioresistance in vitro and in vivo; (iii) reduced miR-203 increases NPC radioresistance through activating IL8/AKT signaling. Our study demonstrates that miR-203 is a critical determinant of NPC radioresoppe, and suggests that targeting the miR-203/IL8/AKT signaling axis is a promising approach for enhancing NPC sensitivity to radiotherapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

Conception and design: J.-Q. Qu, H.-M. Yi, Z.-Q. Xiao
Development of methodology: T. Xiao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.-Q. Qu, H.-M. Yi, X. Ye, J.-F. Zhu, H. Yi, L.-N. Li, T. Xiao, L. Yuan, J.-Y. Li, Y.-Y. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.-Q. Qu, H.-M. Yi, J. Feng, Q.-Y. He, S.-S. Lu
Writing, review, and/or revision of the manuscript: J.-Q. Qu, Z.-Q. Xiao

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