MEDICAL SCIENCE MONITOR

CLINICAL RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 3161-3169
DOI: 10.12659/MSM.915624

Received: 2019.02.11
Accepted: 2019.04.10
Published: 2019.04.29

Improved Radiotherapy Sensitivity of Nasopharyngeal Carcinoma Cells by miR-29-3p Targeting COL1A1 3'-UTR

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Source of support: Departmental sources

Background: Radio-resistance is an obstacle to the treatment of human nasopharyngeal carcinoma (NPC). However, how microRNAs (miRNA) are involved in this process remains unclear. In the present study we explored the role and possible molecular mechanism of miR-29a-3p, formerly known as tumor suppressors, in radio-sensitivity of NPC cells.

Material/Methods: A radio-resistant sub-cell line, CNE-2R, was established to detect the expression of miR-29a/b/c-3p using qRT-PCR. CCK-8 assay, colony formation assay, and single-cell gel electrophoresis (SCGE) assay were carried out to analyze the radio-sensitivity of NPC cells. qRT-PCR, luciferase reporter, and Western blot experiments were performed to validate the targeting of COL1A1 by miR-29a. Short interference RNAs (siRNAs) were used to investigate whether COL1A1 mediates the radio-sensitizer role of miR-29a. Expression of miR-29a and COL1A1 in radio-resistant NPC tissues was finally determined.

Results: miR-29a was decreased in the radio-resistant CNE-2R cells. Following a time-course irradiation (IR) exposure, miR-29a exhibited a time-dependent decrease. Cellular experiments confirmed that miR-29a induced radio-sensitivity of CNE-2R cells via suppressing cell viability and enhancing cell apoptosis after IR. We confirmed that COL1A1 is a direct target of miR-29a and can exert radio-resistance effects in NPC cells. We also found that knockdown of COL1A1 inhibits NPC cell viability and sensitivity to IR. Finally, we observed a downregulation of miR-29a in radio-resistant NPC tissues and its decrease was associated with upregulation of COL1A1.

Conclusions: miR-29a is a critical determinant of NPC radio-response for NPC patients, and its induction provides a promising therapeutic choice to elevate NPC radio-sensitivity.

MeSH Keywords: MicroRNAs • Nasopharyngeal Neoplasms • Radiotherapy, Adjuvant

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/915624

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Background

Nasopharyngeal carcinoma (NPC) is a type of head and neck epithelial malignancy derived from nasopharyngeal epithelial cells, which is prevalent in Southeast Asian countries [1]. The prognosis of NPC patients remains unsatisfactory, especially in advanced NPCs [2]. Radiotherapy is the first-choice treatment for NPC patients. However, about 30% of NPCs develop local recurrence and distant metastasis after radiotherapy due to radioresistance, which can lead to local recurrence and distant metastasis, and is a major obstacle to treatment success in many patients [3,4]. However, the details of the mechanism of NPC radioresistance remains largely unclear. Development of novel effective radiosensitizers will certainly benefit the therapeutic effects for radioresistant NPC patients.

MicroRNAs (miRNAs) are a subclass of short non-coding RNAs (approximately 22 nt in length) that repress gene expression by degradation or translational repression of their target mRNAs through base pairing to the 5'-untranslated region (UTR), 3'-UTR, or coding region of target mRNAs [5,6]. miRNAs are believed to take part in almost all physiological and pathological processes, including cancer development [7]. They also play a critical role in regulating cancer cell radiosensitivity or radioresistance by affecting related processes such as cell cycle, apoptosis, and DNA repair [8,9]. In NPC, several miRNAs, (e.g., miR-138-5p and miR-24) elevate radiosensitivity by targeting important molecules [10,11]. Regulation of radiosensitivity via miRNA-associated mechanisms has recently become a significant research area in NPC [12]. Research also shows that miRNAs are novel targets for improving the radiotherapy of NPC.

The objective of this study was to elucidate the precise function of miR-29a in NPC radiosensitivity. We successfully established a radioresistant CNE-2R sub-cell line. Our data verified that miR-29a introduction enhanced radiosensitivity via suppressing cells viability and inducing cell apoptosis after IR. Additionally, by use of siRNA technology, we found that COL1A1 is a direct target of miR-29a, which mediates miR-29a-induced radiosensitivity in NPC cells. Our results indicate that miR-29a and COL1A1 may be effective targets for overcoming of NPC radioresistance.

Material and Methods

Patients and tissues

We enrolled 34 NPC patients without distant metastasis (M0 stage) when diagnosed who received radiotherapy alone in the Tianjin Medical University General Hospital between Jan 2015 and Dec 2016. Based on the criteria reported previously [13], of the 34 NPC patients, 18 were radiosensitive and 16 were radioresistant. Written informed consent from every patient was obtained prior to participation in the study. NPC tissues were immediately snap-frozen and kept at –80°C until later use for qRT-PCR. The research related to human use complied with all the relevant national regulations and institutional policies and was performed in accordance with the tenets of the Helsinki Declaration, and was approved by the Tianjin Medical University General Hospital's Institutional Review Board or equivalent committee.

Cell cultures

The human undifferentiated non-keratinizing nasopharyngeal carcinoma cell line CNE-2 was obtained from Zhongshan University (Guangzhou, China). A radioresistant cell line was generated as described in a previous report [14]. Briefly, we exposed CNE-2 cells in exponential growth phase to a range of doses of IR (2, 4, 6, and 8 Gy), each delivered 3 times at a dose rate of 100 cGy/min. An interval of 6 to 8 weeks between each IR allowed the surviving cells to regenerate. The whole process of IR and culture lasted for about 1 year, and we refer to the surviving cell line as CNE-2R. CNE-2 and CNE-2R cells were routinely cultured in RPMI-1640 medium (Invitrogen, Life Technology, USA) containing 10% fetal bovine serum (Invitrogen, Life Technology, USA), penicillin 100 units/ml, and streptomycin 100 μg/ml in a humidified atmosphere of 5% CO_2 at 37°C.

Transfection of inhibitors or mimics of miR-29a-3p

Cells were transfected with miR-29a-3p inhibitors or mimics (Ribobio Co., Guangzhou, China) as per the manufacturer's instructions. To ensure the effect of miRNA inhibitors or mimics, transfected cells were surveyed using the fluorescence image system and the content of miR-29a-3p was determined using the qRT-PCR method. COL1A1 siRNAs were purchased from Santa Cruz (sc-44041, Dallas, TX, USA). Transfections were performed by using Lipofectamine® 2000 (Invitrogen, Life Technology, USA) as per the manufacturer's protocol.

Quantitative real-time PCR (QRT-PCR)

qRT-PCR was done to quantify miR-29a/b/c-3p expression using the TaqMan® miRNA reverse transcription kit and TaqMan miRNA assay kits (Applied Biosystems), as per the manufacturer's instructions. Abundance for each miRNA was normalized to U6B rRNA and then calculated by the comparative CT method. The specific primers of miR-29a/b/c-3p were purchased from Ribobio Co. (Guangzhou, China). COL1A1 mRNA expression was also analyzed by qRT-PCR and normalized to β-actin levels.

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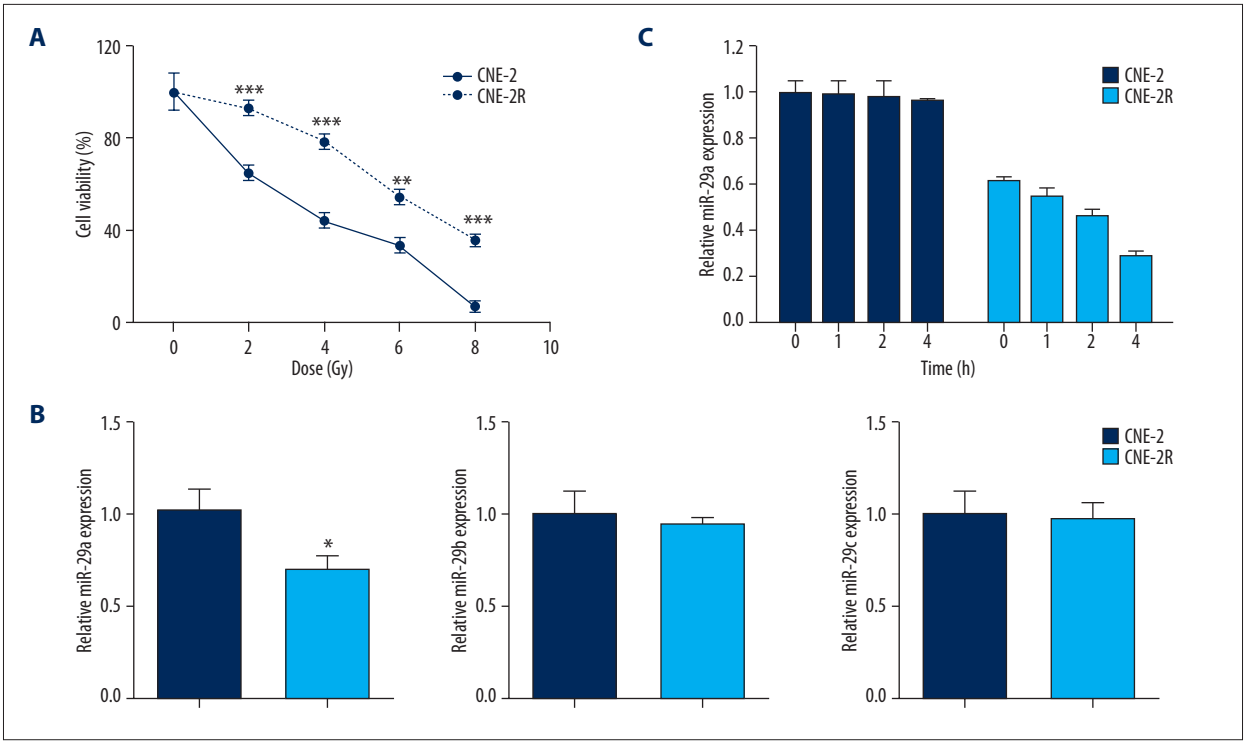

Figure 1. miR-29a is downregulated in radioresistant NPC cells. (**A**) Radioresistance characterization of CNE-2R. CNE-2 and CNE-2R cells were exposed to IR (0, 2, 4, 6, or 8 Gy) every day, and the cell viability was assessed on day 4 by CCK-8 assay. The cell viability ratio (%) is relative to 0 Gy. (**B**) The expression of miR-29a, miR-29b, and miR-29c was analyzed by qRT-PCR in CNE-2 and CNE-2R cells. (**C**) Relative miR-29a expression level is different between CNE-2 and CNE-2R after IR. qRT-PCR was performed to quantify miR-29a expression level in CNE-2 and CNE-2R cells before and after IR. U6B was used for internal controls. * $P<0.05$.

Cell viability assay

CNE-2 or CNE-2R cells were seeded into 96-well plates with a density of 2.5×10^3 cells/well and maintained overnight. Then, the cells were subjected to the indicated doses of IR (0, 2, 4, 6, or 8 Gy) every day using a gamma irradiator. On day 4, cell viability was measured by CCK-8 method (Dojindo, Kumamoto, Japan) by absorbance at 450 nm. Three independent experiments were performed in quadruplicate.

Colony formation assay

Cells were seeded into 6-well plates with a density of 2×10^3 cells/well. Then, the cells received 4 Gy IR radiation dosage and then were cultured for 9 days. The number of colonies/number was counted in each well. All procedures were repeated in triplicate.

Flow cytometry analysis

Cell apoptosis assay was conducted by flow cytometry using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, USA) according to the manufacturer's instructions. Briefly, transfected cells were subjected to doses of IR (0 or 4 Gy) every day using a gamma irradiator. After 96 h, cells were harvested and suspended in binding buffer at a concentration of 2×10^6 cells/ml. Then, 5 µl FITC Annexin V and 5 µl Propidium Iodide Staining Solution were added to 200 µl of cell suspension. The mixture was incubated for 0.5 h in the dark at room temperature. Cells apoptosis was detected by BD FACSVerse™ (BD Biosciences, USA). Apoptosis rates of cancer cells were analyzed by BD FACSuite software.

Bioinformatic analysis

The information and sequence of human miR-29a/b/c-3p was obtained in miRBase (*http://www.miRBase.org/*). The prediction of miR-29a/b/c-3p targets was acquired from the TargetScan program (*http://www.targetscan.org/*).

Statistical analysis

Statistical analyses were performed using SPSS software v19.0 (*http://www-01.ibm.com/software/analytics/spss/*). All graphs were made using GraphPad software v5.0. Data from 3 independent experiments are expressed as mean ±SD. Differences

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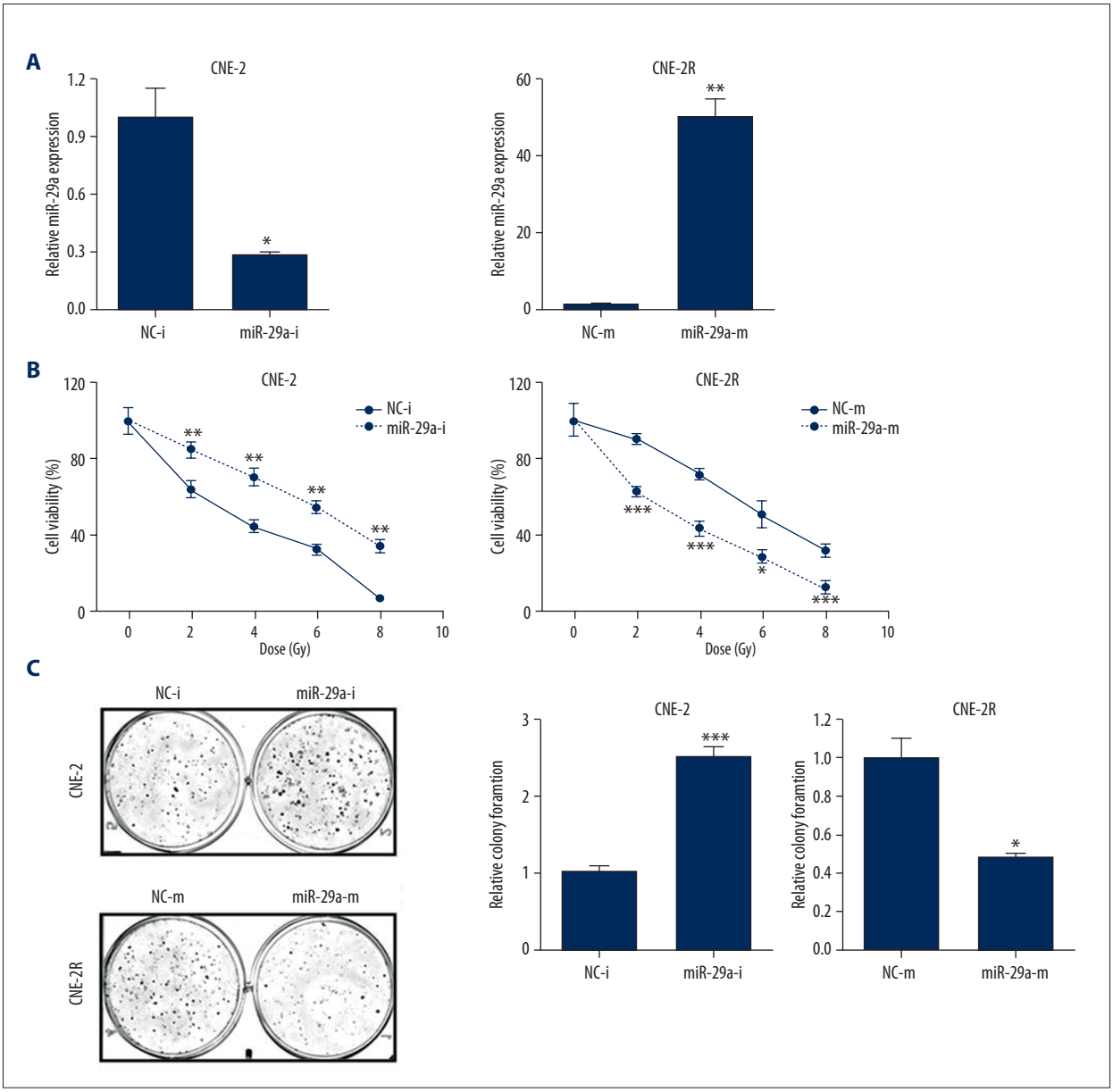
between groups were assessed by the 2-tailed *t* test. The relationship between miR-29a and COL1A1 expressions was assessed by Spearman rank correlation coefficient test. P<0.05 was considered statistically significant.

Results

Validation of miR-29a reduction in NPC radioresistant CNE-2R cells

To investigate the radioresistance mechanisms of NPC cells, we first established a radioresistant CNE-2R sub-cell line by exposing CNE-2 cells to a repeated IR dose of 4 Gy each with 4 rounds of IR. To verify the radioresistant phenotype of CNE-2R cells, we irradiated both CNE-2 and CNE-2R cells with increasing doses of IR (0, 2, 4, 6, and 8 Gy) and examined cell viabilities by CCK-8 assay. As shown in Figure 1A, CNE-2R cells exhibited a significantly stronger viability, in other words, a marked radioresistance, compared with CNE-2 cells. Next, we used qRT-PCR to analyze the expression of miR-29s (miR-29a/b/c-3p) in these radioresistant CNE-2R cells compared with normal CNE-2 cells. Our data clearly showed that miR-29a was obviously decreased in CNE-2R cells, whereas miR-29b and -29c exhibited minor differences between the 2 cell lines (Figure 1B). To further study the effect of IR on miR-29a expression, we exposed CNE-2 and CNE-2R cells to 4 Gy of IR for different time periods. As shown in Figure 1C, the miR-29a level was reduced along



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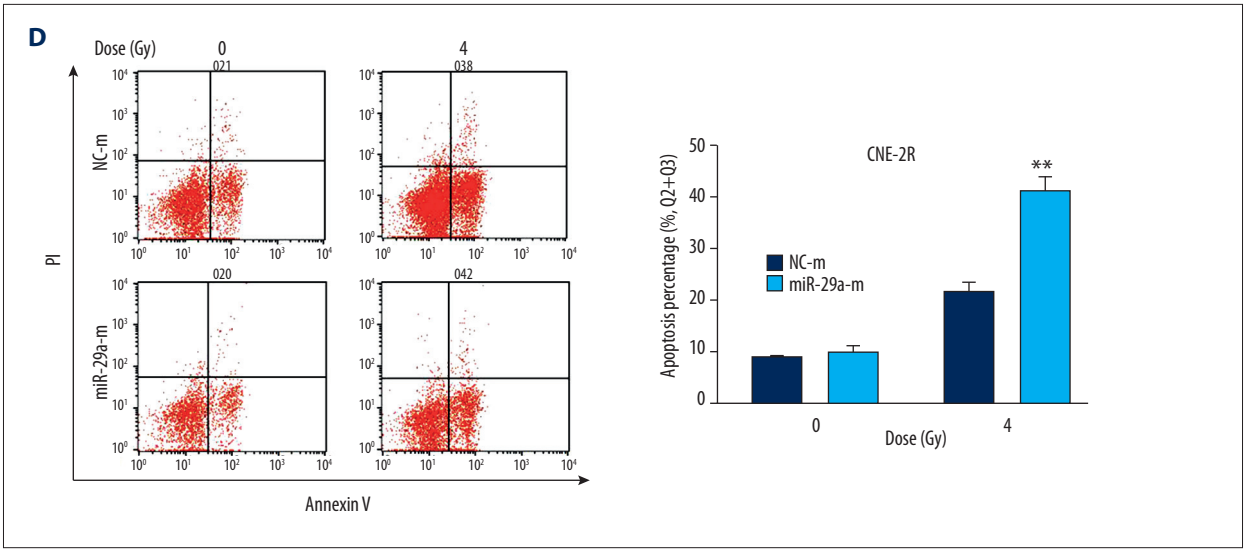

Figure 2. miR-29a enhances radiosensitivity of NPC cells. (**A**) Assessment of transfection efficiency in CNE-2 and CNE-2R cells transfected with miR-29a inhibitors or mimics by qRT-PCR. (**B**) Transfection of miR-29a inhibitors in CNE-2 cells increased cell viability after exposure to irradiation, while transfection of miR-29a mimics in CNE-2R cells significantly inhibited cell viability. CNE-2 cells transfected with control or miR-29a inhibitors and CNE-2R cells transfected with control or miR-29a mimics were irradiated every day with a range of 0 to 8 Gy radiation doses, and the cell viability was assessed on day 4 by CCK-8 assay. (**C**) Colony formation assay was performed in CNE-2 and CNE-2R cells exposed to 4 Gy IR after transfection with miR-29a mimics or inhibitors. (**D**) Flow cytometry analysis of cell apoptosis. miR-29a mimics transfection dramatically elevated the apoptotic levels of CNE-2R cells when exposed to irradiation (0 or 4 Gy). Top left quadrant (Q1) indicates non-apoptotic cells; top right quadrant (Q2) represents late apoptosis events; bottom right quadrant (Q3) represents early apoptosis cells; and bottom left quadrant (Q4) represents living cells. Each experiment was performed in triplicate independently. * $P<0.05$; *** $P<0.001$.

with lasting IR exposure in CNE-2R but remained constant in CNE-2 cells, suggesting that miR-29a is an IR-responsive miRNA in CNE-2R cells.

miR-29a suppresses NPC cell viability and sensitizes NPC cells to IR

To characterize the role of miR-29a in NPC radioresistance, we transiently transfected CNE-2 cells with control or miR-29a inhibitor, or transfected CNE-2R cells with control or miR-29a mimic, then evaluated radiosensitivity by analyzing cell viability and apoptosis. The successful transfection efficiency and specificity were confirmed by qRT-PCR (Figure 2A). We found that CNE-2 cells transfected with miR-29a inhibitor exhibited enhanced cell viability, whereas CNE-2R cells transfected with miR-29a mimic exhibited significantly suppressed cell viability after IR exposure compared with the control groups, revealed by both CCK-8 and colony formation assays (Figure 2B, 2C). Moreover, we assessed the role of miR-29a in IR-induced cell apoptosis of CNE-2R. Cell apoptosis assay found that miR-29a strengthened IR-induced apoptosis of CNE-2R cells compared with the control group (Figure 2D). To summarize, our findings confirmed that increasing the miR-29a level markedly sensitized radioresistant NPC cells to IR.

miR-29a targets COL1A1 3'UTR

To better understand the mechanism of miR-29a-guided NPC radiosensitivity, we next searched for potential targets of miR-29a using prediction tools, including Targetscan. Among the numerous predicted targets, we focused on the collagen type I alpha 1 (COL1A1) gene. We found that the 3'-UTR of the human COL1A1 gene contains a miR-29a/b/c binding site (Figure 3A). By constructing luciferase reporters containing wild-type or mutant miR-29a target sequences of the COL1A1 3'-UTR, we found that miR-29a mimic transfection repressed the luciferase activity of wild-type COL1A1 3'-UTR, while miR-29a inhibitors transfection enhanced it (Figure 3B). However, it exerted minor only a slight effect on the mutant reporter (Figure 3B). qRT-PCR and immunoblotting detection further confirmed that miR-29a targeted COL1A1 mRNA and protein expression (Figure 3C, 3D). These results indicated that miR-9a represses COL1A1 gene expression post-transcriptionally. Considering the regulatory effects of COL1A1 on apoptosis induced by IR in cervical cancer cells [15], we believe that it might mediate the radio-sensitivity of miR-29a in NPC.

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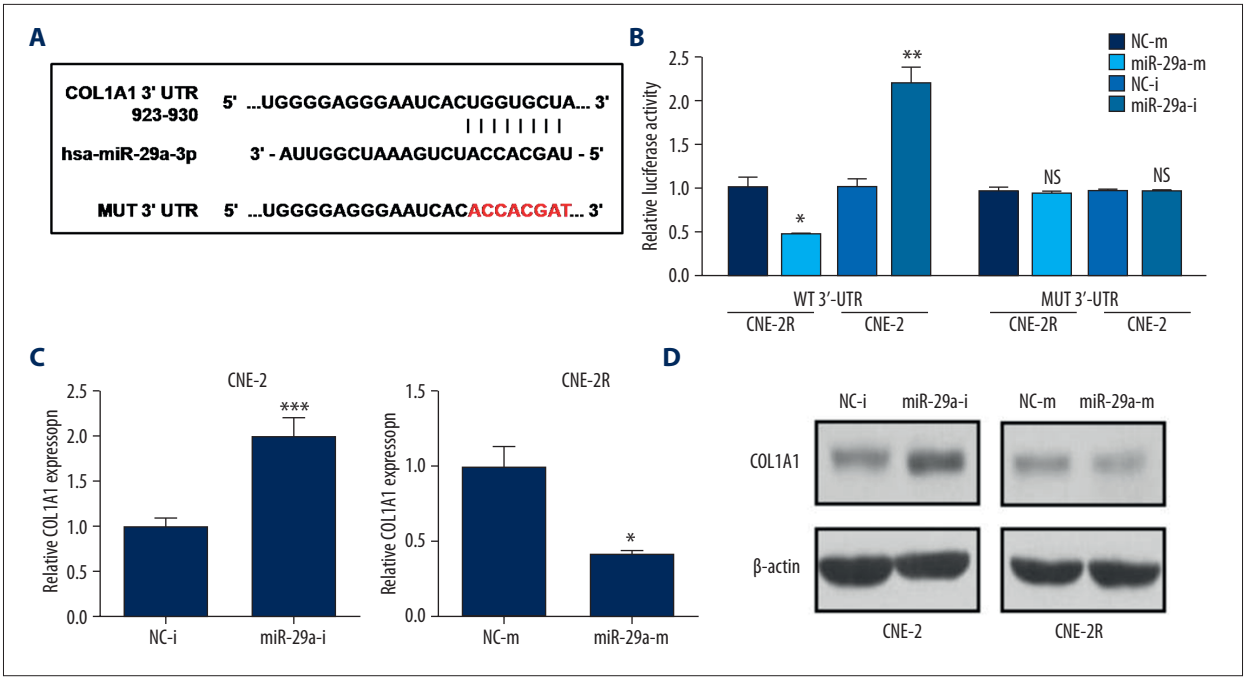

Figure 3. miR-29a-3p directly targets COL1A1. (**A**) The 3'UTR of COL1A1 contains 1 predicted miR-29a-3p-binding site. The mutagenesis nucleotides are indicated in red. (**B**) Dual luciferase reporter assay. CNE-2R cells were transfected with wild-type (WT) 3'UTR-reporter or mutant (MUT) 3'UTR-reporter together with control, miR-29a-3p mimics, or inhibitor. Relative firefly luciferase expression was normalized to Renilla luciferase. (**C**) qRT-PCR was used to examine COL1A1 mRNA level in CNE-2 cells transfected with control or miR-29a-3p inhibitors and CNE-2R cells transfected with control or miR-29a-3p mimics. (**D**) Protein expression analysis of COL1A1 and β-actin was performed by immunoblotting in CNE-2 and CNE-2R cells treated as shown in (**C**). Data are presented as mean±SD from 3 independent experiments. * $P<0.05$; ** $P<0.01$.

Knockdown of COL1A1 inhibits NPC cell viability and sensitizes NPC cells to IR

To investigate whether COL1A1 can mediate the radio-sensitizer role of miR-29a, we then examined the specific effects of COL1A1 in NPC cells. qRT-PCR revealed that in the radioresistant CNE-2R cells, COL1A1 mRNA level was significantly upregulated compared with that in the normal CNE-2 cells (Figure 4A). SiRNAs against COL1A1 were then used to knock down the endogenous COL1A1 (Figure 4B). Consistently, after IR exposure, CNE-2R cells exhibited reduced cell viability, decreased colony formation, and enhanced apoptosis upon COL1A1 knockdown (Figure 4C–4F). Therefore, these results suggested that upregulated COL1A1 is closely correlated with the radioresistence phenotype of CNE-2R cells.

Decreased miR-29a is associated with NPC radioresistance and COL1A1 upregulation

Finally, we analyzed the expression of miR-29a and COL1A1 in NPC tissues, including 18 radiosensitive and 16 radioresistant NPCs. The results showed that miR-29a was decreased in the radioresistant NPCs (Figure 5A). In contrast, COL1A1 mRNA levels were upregulated in the 16 tested radioresistant NPCs (Figure 5B). Furthermore, we found a negative correlation of expression in radiosensitive NPCs and radioresistant NPCs (Figure 5C, 5D). These findings are consistent with the findings shown above in Figure 1 and validate the true targeting of COL1A1 by miR-29a in NPC.

Discussion

Radioresistance largely restricts the successful treatment of human NPC. Investigation of the underlying mechanisms involved in the acquisition of radioresistance is crucial to improve NPC patients' outcomes. Also, identification of novel molecules enhancing radiosensitivity provides promising strategies for its therapy. The mechanisms governing cancer cell radioresistance have been extensively explored by establishing radioresistant cell models [16,17].

Using high-throughput methods, many mRNAs and miRNAs have been found to be aberrantly expressed between radioresistant and radiosensitive NPC cell lines [18]. The role of miRNAs in radiosensitivity provides an important focus to overcome radioresistance of cancer. For instance, miR-214 and miR-195 are strongly involved in the development of radioresistance in

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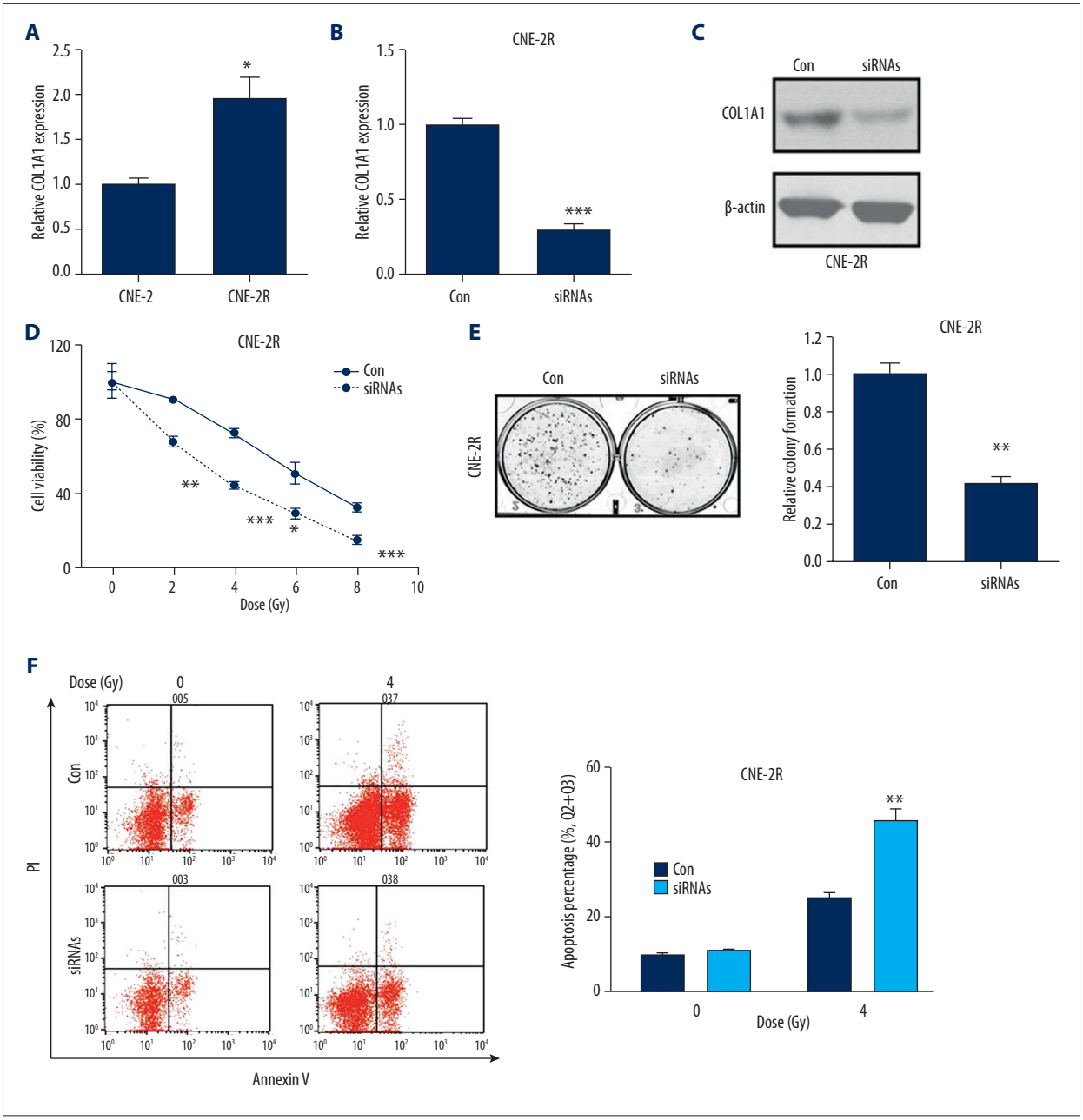

Figure 4. Knockdown of COL1A1 inhibits cell viability and sensitizes NPC cells to IR. (**A**) The expression of COL1A1 was analyzed by qRT-PCR in CNE-2 and CNE-2R cells. (**B**) Assessment of knockdown efficiency in CNE-2R cells transfected with COL1A1 siRNAs by qRT-PCR. (**C**) Assessment of knockdown efficiency in CNE-2R cells transfected with COL1A1 siRNAs by immunoblotting. (**D**) Transfection of COL1A1 siRNAs in CNE-2R cells significantly inhibited cell viability after exposure to irradiation. Transfected cells were irradiated every day with a range of 0 to 8 Gy radiation doses, and the cell viability was assessed on day 4 by CCK-8 assay. (**E**) Colony formation assay was performed in CNE-2R cells exposed to 4 Gy IR after transfection with COL1A1 siRNAs. (**F**) Flow cytometry analysis of cell apoptosis. COL1A1 siRNAs transfection dramatically elevated the apoptotic levels of CNE-2R cells when exposed to irradiation (0 or 4 Gy). Top left quadrant (Q1) indicates non-apoptotic cells; top right quadrant (Q2) represents late apoptosis events; bottom right quadrant (Q3) represents early apoptosis cells; and bottom left quadrant (Q4) represents living cells. Each experiment was performed in triplicate independently. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

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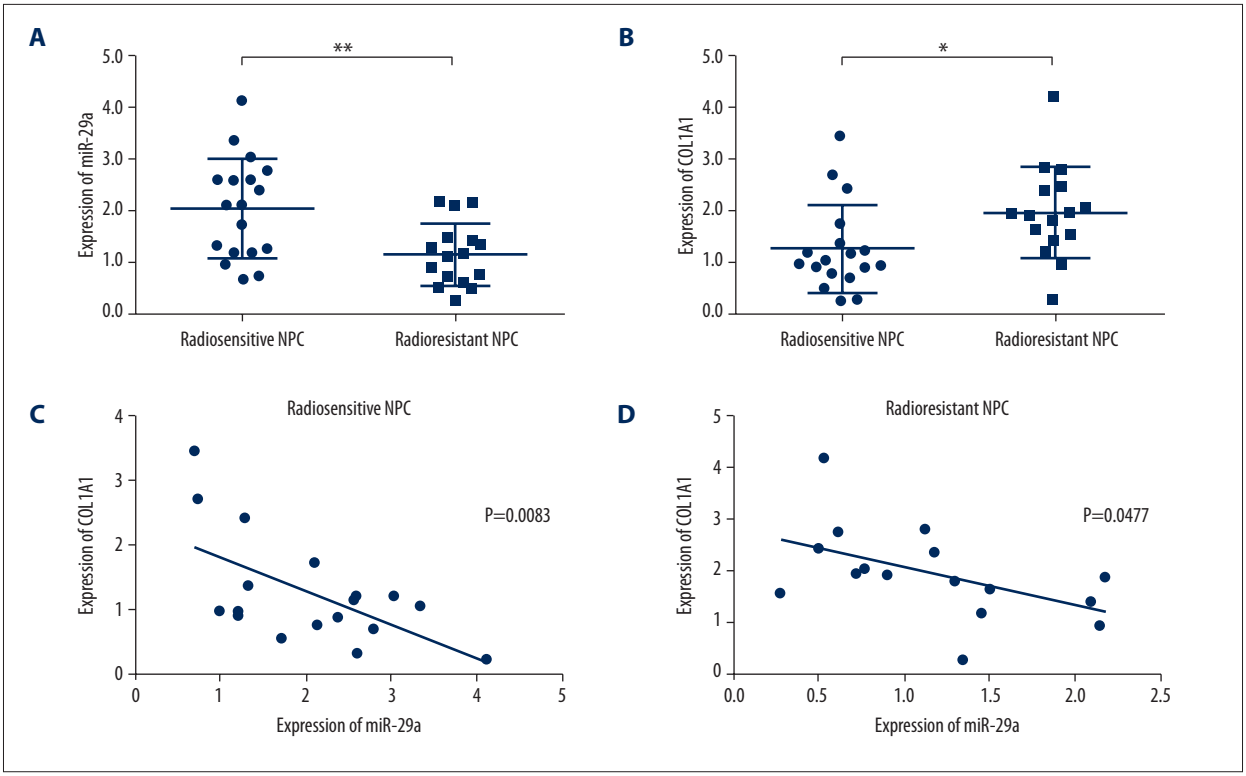

Figure 5. Correlation of miR-29a and COL1A1 expression levels with NPC radio-resistance. (**A**) qRT-PCR was performed to determine the expression levels of miR-29a in the radiosensitive and radio-resistant NPC tissues. (**B**) qRT-PCR was performed to determine the expression levels of COL1A1 in the radiosensitive and radio-resistant NPC tissues. (**C**) The COL1A1 mRNA expression levels were negatively correlated with the expression levels of miR-29a in radiosensitive NPC tissues. (**D**) The COL1A1 mRNA expression levels were negatively correlated with the expression levels of miR-29a in radioresistant NPC tissues. * $P<0.05$; ** $P<0.01$.

certain cancers [19,20]. In NPC, many lines of evidence have shown that miR-138-5p and miR-24 elevate radiosensitivity by targeting important molecules [10,11].

MiR-29s (including miR-29a, -29b, and -29c) are known to critically affect cancer progression by functioning as tumor suppressors [21]. However, their roles in NPC remain poorly understood. There are only 2 reports showing the contradictory functions of miR-29s in NPC: Qiu and colleagues found that miR-29a/b overexpression had no significant effect on cell proliferation but they could enhance cell migration and invasion in NPC cells [22]; however, Gao et al. described a critical role of miR-29a in mediating Taxol sensitivity and showed that miR-29a overexpression inhibited proliferation and induced apoptosis in NPC cells [23]. There has been little research on the involvement of miR-29a in cancer radiosensitivity. Nevertheless, there are some clues indicating the role of miR-29a in mediating radiosensitivity. In colorectal cancer, miR-29a was verified to enhance radioresistance via activating the PI3K/Akt pathway [24]. However, another large-scale *in vivo* loss of function screen identified miR-29a as a modulator of radiosensitivity, since loss of miR-29a led to enhanced clonogenic survival and reduced apoptosis in irradiated tumor cells [25]. In the present study we established a radioresistant CNE-2R sub-cell line following standard procedures. Surprisingly, we found that miR-29a but not miR-29c was decreased in this radioresistant CNE-2R sub-cell line. Subsequent function assays further characterized the role of miR-29a in regulating radiosensitivity of NPC cells. Our findings support that miR-29a is a potent radio-sensitizer in NPC cells. The inconsistent functions of miR-29a in different cancers might be context-specific outcomes.

miRNAs exert their functions mainly through the targeting of downstream gene expression. Here, we showed that COL1A1, encoding the subunit of type I collagen, is a target of miR-29a. Actually, this targeting has been reported by several other groups [26,27]. Collagen is the main protein of bones, tendons, and teeth, and participates in cancer cell adhesion, gap junction, and extracellular matrix (ECM). Its involvement in radioresistance was only recently reported in cervical cancer cells [15]. By inhibiting apoptosis, COL1A1 can modulate the radioresistance of cervical cells via complex mechanisms involving Caspase-3/PI3K/AKT pathways [15]. Here, we provide evidence that COL1A1 itself can enhance cell viability, colony

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formation, and radioresistance in NPC cells, since knockdown of COL1A1 resulted in the opposite effects. However, the precise mechanisms of COL1A1 in NPC radioresistance need to be explored in the near future.

Conclusions

Taken together, our results indicate that miR-29a is decreased in NPC radioresistant cells and tissues, and miR-29a can directly target the 3'-UTR of the COL1A1 gene and lead to radiosensitivity in NPC cells. However, the potential applications of miR-29a and COL1A1 as molecular biomarkers and therapeutic targets for NPC treatment require further investigation.

Conflict of interest

None.

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