Abstract. Osteosarcoma is a kind of high-risk sarcoma of the skeleton typically observed in people under 25 years old. Currently, radiotherapy is widely applied in cancer treatment. However, osteosarcoma is radioresistant and accordingly new, more effective radiosensitizers are needed. miRNAs have been reported to play an important role in osteosarcoma radiosensitivity. We examined the modulating effect of miR-328-3p in vivo and in vitro. miR-328-3p was downregulated in HOS-2R cells. The overexpression of miR-328-3p enhanced the radiosensitivity of osteosarcoma cells. miR-328-3p inhibited proliferation and promoted apoptosis in osteosarcoma cells under radiation conditions. In cells overexpressing miR-328-3p, H2AX expression was downregulated. We found that miR-328-3p targets H2AX and inhibits its expression. It was concluded, that miR-328-3p enhances the radiosensitization of osteosarcoma following X-ray irradiation, and determined that it directly targets H2AX to regulate radiosensitization.

Introduction

Osteosarcoma (OS) originates from the metaphyseal areas of the long bones and is a frequently occurring disease in children, adolescents, and young adults (1,2). The risk of developing OS is highest among people under 25 years old and second-highest in individuals aged 60-80 years (3,4). Before the 1970s, the mortality rate was ~80% because surgery was the only method for OS treatment. With advances in surgical techniques and the emergence of chemotherapy, the disease-free survival rate has improved substantially (5,6). In response to tumor metastasis, radiotherapy has emerged as a more efficient treatment (7,8). However, radiotherapy to treat OS is limited by radioresistance (9,10). Therefore, it is necessary to study the molecular mechanism of OS radioresistance to improve the efficiency of OS radiotherapy. Ionizing radiation can activate reactive oxygen species (ROS) and cause oxidative damage to DNA bases. DNA double-strand breaks (DSB) have a dual influence on tumor therapy; they can lead to cancer, but can also kill cancer cells. The phosphorylation of histone H2AX is a sensitive marker for DSB (11) and H2AX contributes to DSB repair (12). The expression level of phospho-H2AX (pH2AX) contributes to the detection of epithelial ovarian cancer (13). γH2AX plays an important role in the response to DSB and participates in the DSB repair process mediated by DNA repair enzymes (14).

MicroRNAs (miRNAs) are short (18-24 nucleotides) single-stranded non-coding RNAs; they cannot be translated into proteins or oligopeptides. In some cases, miRNAs can post-transcriptionally regulate gene expression by binding to the target site in mRNA (15,16). Some studies have found that miRNAs are involved in the process of tumor development. The overexpression of miR-187 can inhibit colorectal cancer progression by directly targeting CD276 (17). In OS, miR-489-3p is downregulated and the overexpression of miR-489-3p can suppress OS progression by targeting PAX3 (18). Recent research has indicated that miRNAs are also involved in tumor radioresistance. miR-185 can target ATR (rad3-related), which can respond to DNA damage and DNA replication stress, enhancing radiosensitivity in tumor cells (19). In esophageal squamous cell carcinoma, the expression level of miR-98 is positively correlated with radiosensitivity (20). In cervical cancer cells, miR-424 can target aprataxin and the overexpression of miR-424 can increase the cell sensitivity to radiation (21).

In our previous study, miR-328-3p expression was distinctly reduced in a radiation-tolerant HOS cell line (HOS-2R) (Fig. 1). We hypothesized that miR-328-3p plays a role in the development of OS cells. In the present study, we examined the correlation between miR-328-3p and radioresistance in OS cells in vivo and in vitro. We determined the regulatory role of miR-328-3p in OS radioresistance by overexpressing or knocking out miR-328-3p in OS cell lines, and we showed that miR-328-3p targets H2AX to enhance OS radiosensitivity.

Materials and methods

Cell culture, irradiation, and transfection. The human osteosarcoma cell line HOS was purchased from the Cell Culture Center, Institute of Basic Medical Sciences (Beijing, China).
U2-OS cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cultures of the cell lines were maintained in a humidified, 37°C, 5% CO₂ incubator. McCoy’s 5A Media (modified with Tricine) (Sigma, St. Louis, MO, USA) with 10% FBS (Gibco, Grand Island, NY, USA) was used for cell culture. The cells in exponential growth were exposed to X-ray radiation at 2, 4 or 8 Gy at room temperature to measure radioresistance in vitro. An X-ray machine (Faxitron RX-650; Tucson, AZ, USA) with 100 kVp (0.835 Gy/min) was used for irradiation.

Cells were transfected with miRNA mimics and a control, i.e., miR-328-3p mimics, miR-328-3p ASO (anti-miRNA oligos), or ASO control (RiboBio, Guangzhou, China). Additionally, siRNA-H2AX and siRNA control were purchased from GeneChem (Shanghai, China).

Establishment of radioresistant cell lines. HOS cells (2x10⁷) were cultured in McCoy’s 5A Media supplemented with 10% FBS. Ten minutes before irradiation, the cells were replaced with fresh medium, then cells were irradiated by an X-ray machine (0.835 Gy/min) with 2 Gy dose. To obtain radioresistant cell population, a total dose of 44 Gy was reached by 22 fractions of irradiation. Each time after irradiation, the culture medium was replaced with fresh complete medium. When the cell confluence reached >80%, the cells were subcultured. Additionally, the next irradiation was performed when the cell confluence reached 50%.

Quantitative real-time PCR. Total RNA was extracted from OS cell lines using TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using the TaqMan miRNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA). Then, the cDNA was amplified for 28 cycles in a PCR machine (Roche): 94°C for 30 sec; annealing for 30 sec, 72°C for 30 sec. Quantitative real-time PCR (qRT-PCR) analyses were performed with SYBR® Premix Ex Taq™ (Takara, Japan) using a StepOne-plus Real-Time PCR System (Applied Biosystems). The PCR cycling was as follows: 95°C for 2 min, 40 cycles of 95°C for 10 sec, 60°C for 20 sec, 72°C for 20 sec. The relative expression levels of miRNAs were calculated using the 2^{-ΔΔCt} method. U6 snRNA was used as internal controls to normalize the expression levels of miRNAs. The qPCR primers for miR-328-3p were as follows: F:5’-TGGCGCTGACCCTCTCTGCCG-3’; R:5’-CCAGTGC AGGGTCCGAGGT-3’. The qPCR primers for U6 snRNA were as follows: F:5’-TGGCGGTGAGCCCTCTCTGCCG-3’; R:5’-CCAGTGCAGGGTCCGAGGT-3’.

MTT assay. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl- tetrazolium bromide (MTT) assay was performed to measure the viability of osteosarcoma cells using the Cell Proliferation Kit I (Sigma), following the procedure described in the kit manual. Cells were grown in 96-wells in a final volume of 100 µl of culture medium per well in a humidified 37°C incubator. MTT labeling reagent was added to each well to obtain a final concentration of 0.5 mg/ml. Samples were incubated for 4 h in a humidified atmosphere. Then, 100 µl of solubilization solution was added to each well, followed by incubation (37°C) overnight. Absorbance of the samples was measured spectrophotometrically using a microplate reader.

Western blot analysis. Cells were scraped from the wells after washing twice with cold PBS. Total proteins were extracted using ice-cold RIPA lysis buffer (Solarbio, Beijing, China) with a protease inhibitor. Then, 40 µg of total proteins from each sample was subjected to 12% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After samples were washed and blocked with 5% non-fat milk, the membrane was incubated with the primary antibodies (Abcam, UK). After they were washed in TBS, the membrane was treated with a horseradish peroxidase-conjugated secondary antibody (ICLLAB, USA). Proteins were observed by chemiluminescence (Millipore, Billerica, MA, USA).

Survival colony formation assay. Cell suspensions were exposed to X-ray radiation; then, 1x10⁶ cells were seeded in 6-well plates and cultured for 12 days. Detailed cultivation methods are described above (Cell culture and irradiation). The number of colonies that contained at least 50 cells was counted.

Apoptosis. Cells (1x10⁶) were washed with cold PBS and fixed in cold 70% ethanol overnight at -20°C. The Annexin V-FITC Apoptosis Detection kit (Abcam) was used to perform apoptosis assays following the manufacturer's instructions. The samples were analyzed using the FACS III (BD Biosciences, Franklin Lakes, NJ, USA).

Tumor xenograft assay. Female nude mice (BALB/c, 5 weeks old) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China) and housed under SPF conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee of Guizhou Provincial People's Hospital (Guizhou, China).

The mouse tumor xenograft model was established; 1x10⁷ HOS cells were suspended in 0.1 ml of PBS and subcutaneously injected into the right thigh of the nude mice. Ten days later, the mice were intratumorally injected with 5 nmol miR-328-3p agomir or 5 nmol miR-NC agomir at days 12, 16, 20, 24, 28 and 32. Mice were exposed to 4 Gy of X-ray radiation after each intratumoral injection. At day 34, the mice were sacrificed by cervical dislocation. The tumor weight was measured using electronic scales (Sartorius, Beijing, China), and the tumor volume was calculated as length x (width)²/2.

Statistical analysis. One-way ANOVA was performed to determine the statistical significance of differences between groups. The numerical results are presented as means ± standard deviation. Differences with a p-value of <0.05 were considered statistically significant.

Results

Modulation of miR-328-3p alters the radiosensitivity of osteosarcoma cells. HOS and U2OS are human osteosarcoma cell lines, we used these two kinds of cells to explore the effect of miR-328-3p on the radiosensitivity of osteosarcoma cells. In order to investigate the relationship between miR-328-3p and radiosensitivity in OS cells, we also established radiation-tolerant HOS cell lines (HOS-2R) by X-ray (2 Gy). miR-328-3p levels in HOS-2R cells were detected by qPCR and the
expression levels were downregulated compared with those in control cells (Fig. 1A). To explore whether the modulation of miR-328-3p affects the radiosensitivity of OS cells, we also performed an MTT assay to determine the survival rate of HOS-2R cells, HOS cells, and U2OS cells following irradiation at 0 or 8 Gy. The overexpression of miR-328-3p reduced HOS-2R cell viability following 8 Gy of X-ray, and there was no significant difference between miRNA control and miR-328-3p mimic groups following irradiation with 0 Gy (Fig. 1B). Then, we silenced miR-328-3p in HOS and U2OS cells using antisense oligonucleotides (ASO). The deletion of miR-328-3p increased the viability of HOS cells following 8-Gy irradiation (Fig. 1C), similar to the results obtained for U2OS cells (Fig. 1D). These results confirmed that miR-328-3p has a regulatory role in OS cell radiosensitivity.

miR-328-3p increases the rate of apoptosis and reduces the proliferation ability of osteosarcoma cells after ionizing radiation. Based on the observation that the abnormal expression of miR-328-3p alters the radiosensitivity of OS cells, we examined whether miR-328-3p influences the apoptosis and proliferation of OS cells. Radioresistant cell lines were established under the condition of 2-Gy irradiation, so we need to increase the dose of X-ray to 4 Gy. Compared to HOS-2R, HOS and U2OS was more sensitive to irradiation. 2 Gy X-ray exposure was enough to investigate the biological characteristics of HOS and U2OS cells. miR-328-3p was overexpressed in HOS-2R cells, and the number of surviving clones after X-ray exposure at 0 or 4 Gy was counted. The surviving clone number for HOS-2R cells overexpressing miR-328-3p decreased following irradiation at 4 Gy (Fig. 2A). Then, we inhibited the expression of miR-328-3p in HOS and U2OS cells using miR-328-3p ASO. The deficiency of miR-328-3p increased the survival rate after X-ray exposure at 2 Gy, both in HOS and U2OS cells (Fig. 2B). Apoptosis was analyzed by flow cytometry following miR-328-3p overexpression or knockout in various cell types (including HOS-2R, HOS and U2OS cells). The overexpression of miR-328-3p enhanced HOS-2R cell apoptosis after exposure to 4 Gy (Fig. 2C). In HOS and U2OS cells in which miR-328-3p was knocked out, apoptosis decreased after exposure to 2 Gy (Fig. 2D). These results confirmed that miR-328-3p inhibits proliferation and promotes apoptosis in OS cells under radiation conditions, and miR-328-3p enhances the radiosensitivity of OS.

miR-328-3p targets H2AX and inhibits its expression. Using the bioinformatics tool TargetScan, we determined that H2AX is a predicted target for miR-328-3p. As shown in Fig. 3A, the 3'-UTR region of H2AX contains a sequence that matches the specific binding sequences of miR-328-3p. A luciferase reporter assay was used to further prove the targeting of miR-328-3p to the H2AX 3'-UTR. The cotransfection of miR-328-3p mimics with the wild-type vector reduced luciferase activity in HOS cells compared to that of control-treated cells, and a similar trend was found in U2OS cells (Fig. 3B). No statistically significant differences in luciferase activity were observed for cells cotransfected with miR-328-3p mimics and mutant vector compared to control-treated cells (Fig. 3B). We also detected the expression levels of H2AX in HOS-2R and HOS cells by western blotting, and HOS-2R cells had a higher level of H2AX expression compared to that of control-treated cells (Fig. 3C), in contrast to the miR-328-3p expression patterns (Fig. 1A). To explore the miR-328-3p regulatory effect on H2AX, we used HOS and U2OS cell lines. We detected the expression levels of H2AX by western blotting after overexpressing or silencing miR-328-3p. We found that the expression of H2AX was suppressed after miR-328-3p mimic transfection in HOS cells. When miR-328-3p was knocked out using miR-328-3p ASO,
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The expression of H2AX increased. A similar phenomenon was observed in U2OS cells (Fig. 3D). These results showed that miR-328-3p targets H2AX, and the modulation of miR-328-3p affects the expression of H2AX.

miR-328-3p mediates the radiosensitivity of osteosarcoma cells by targeting H2AX. We knocked out H2AX using H2AX siRNA in the HOS and U2OS cell lines. We verified the knockout efficiency by western blotting (Fig. 4A). The survival rate of H2AX-deficient HOS cells was reduced following irradiation at 2 Gy compared to that of sham-treated cells (Fig. 4B). A similar trend was also found in U2OS cells (Fig. 4B). To explore the effect of H2AX expression changes on apoptosis in OS cell lines, we examined apoptosis using a flow cytometry assay. Under radiation conditions of 2 Gy, H2AX deletion increased apoptosis in both HOS and U2OS cells (Fig. 4C). We also found that deleting H2AX reduced cell viability following irradiation at 2 Gy in both HOS and U2OS cells (Fig. 4D). Taken together, these results showed that deleting H2AX increases the radiosensitivity of OS cell lines.

To further study the function of miR-328-3p targeted to H2AX, we detected the cell sensitivity to radiation by western blotting. Cells were transfected with ASO con (control), miR-328-3p ASO, miR-328-3p ASO+siRNA con or miR-328-3p ASO+H2AX siRNA, respectively. The expression levels of miR-328-3p were reduced in miR-328-3p ASO transfected, miR-328-3p ASO and siRNA con cotransfected, and miR-328-3p ASO and H2AX siRNA cotransfected HOS cells, similar to the results observed in U2OS cells (Fig. 5A). The expression levels of H2AX increased after silencing...
Figure 3. miR-208b targets H2AX and represses H2AX expression by binding to its 3'-uTR. (A) The 3'-UTR region of H2AX contains a sequence that matches the specific binding sequences of miR-328-3p. (B) The targeting effect of miR-328-3p on H2AX 3'-uTR was detected by a luciferase reporter assay in HOS and U2OS cells. (C) The expression levels of H2AX in HOS and HOS-2R cells were determined by western blotting. (D) The expression levels of H2AX in HOS and U2OS cells were determined by western blotting after overexpressing or silencing miR-328-3p. *p<0.05.

Figure 4. H2AX downregulation reduces radioresistance in osteosarcoma cells. (A) To silence H2AX, si-H2AX was used, and the knockout efficiency of H2AX was detected by western blotting. (B) The survival rate of osteosarcoma cells with or without H2AX silencing after irradiation at 2 Gy. (C) The apoptosis assay was performed by flow cytometry following 2 Gy X-ray irradiation. (D) Cell viability assay was performed in si-control and si-H2AX cells after 2 Gy X-ray irradiation. *p<0.05.
miR-328-3p in both HOS and U2OS cells (Fig. 5B). The deficiency of miR-328-3p decreased apoptosis after 2-Gy X-ray exposure in both HOS and U2OS cells (Fig. 5C). The effect of miR-328-3p knockout on reducing apoptosis was weakened after knocking out miR-328-3p and H2AX simultaneously (Fig. 5C). The deficiency of miR-328-3p increased the viability of both HOS and U2OS cells after 2-Gy X-ray irradiation (Fig. 5D). The enhanced cell viability after miR-328-3p knockout was weakened after knocking out miR-328-3p and H2AX simultaneously (Fig. 5D). These results showed that miR-328-3p targets H2AX and thereby miR-328-3p can participate in the regulation of OS radiosensitivity.

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**Figure 5.** miR-328-3p regulates the radiosensitivity of osteosarcoma cells by targeting H2AX. (A) The expression levels of miR-328-3p were detected by qPCR in HOS and H2OS cells cotransfected with ASO control, miR-328-3p ASO, miR-328-3p ASO+si-control, and miR-328-3p ASO+si-H2AX. (B) The expression levels of H2AX were detected by western blotting after silencing miR-328-3p in HOS and H2OS cells. (C) Apoptosis was detected by flow cytometry in HOS and H2OS cells. (D) Cell viability was examined by MTT assays in HOS and H2OS cells. *p<0.05.
Overexpression of miR-328-3p enhances the radiosensitivity of osteosarcoma in mice. A xenograft tumor model was examined to further study the effect of miR-328-3p on the radiosensitivity of OS in vivo. To test tumor growth following irradiation at 4 Gy, we subcutaneously injected miRNA control transfected HOS-2R cells or miR-328-3p transfecte...
tion of H2AX and enhance the expression of GADD45γ and MDC-1 after UV exposure, and STAT3 reduces DNA damage caused by UV (39). The phosphorylation of H2AX promotes NBS1 expression at damage sites, and the acetylation of histone H2AX at Lys 5 is crucially important for the damaged chromatin-specific binding of NBS1 (40).

We proved that miR-328-3p target to the H2AX 3′-UTR. Previous studies have shown that H2AX is regulated by upstream miRNAs. For example, miR-138 directly targets the histone H2AX to modulate the DNA damage response (41). Lin28 targets H2AX, and a deficiency of Lin28 increases radiosensitivity in breast cancer cells (42). H2AX has one more upstream regulatory element: miR-328-3p. In addition, a negative correlation was found between H2AX expression and miR-328-3p level in HOS-2R cells. This tendency provided an indirect proof that H2AX is a target of miR-328-3p.

One limitation of our study was the inability to obtain clinical specimens for further verification of the effect of miR-328-3p in humans. We tested xenograft tumor growth in mice. The results are consistent with the experiments in vivo. In general, miR-328-3p is a positive regulatory factor in OS radiosensitivity by H2AX, and it is worthwhile to study the function of miRNAs to treat cancer by regulating downstream elements. miR-328-3p shows promise as a new potential therapy target in OS.

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