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Original Article

miR-222 enhances radiosensitivity of cancer cells by inhibiting the expression of CD47

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Abstract: Radiotherapy is one of the most common and effective treatments for localized cancer. However, radiotherapy kills tumor cells while causing damage to surrounding normal cells. Enhancing the radiation sensitivity of tumor cells and reducing the radiation damage to normal cells is a difficult problem. Here, we find that the expression of a human microRNA (miRNA), hsa-miR-222, is upregulated in response to ionizing radiation. TargetScan analysis shows that the 3' UTR of CD47 is potentially targeted by miR-222. This prediction was validated by luciferase reporter and mutation assays. It was demonstrated that miR-222 negatively regulates CD47 expression at mRNA and protein levels, and overexpression of the miR-222 enhances cancer cell radiosensitivity by the CD47-pERK pathway in cancer cells. Our findings enrich the complex relationship between miRNA and CD47 in irradiation stress and shed light on the potential of miRNAs both for direct cancer therapeutics and as tools to sensitize tumor cells to radiotherapy.

Keywords: miR-222, CD47, radiosensitivity, ERK pathway

Introduction

Irradiation is a primary mode of cancer therapy for over half of all cancer patients, nearly 60% of new diagnosed cancer patients receiving some form of radiation therapy [1]. Ionizing radiation can cause DNA damage in tumor cells, which in turn induces apoptosis in tumor cells for therapeutic purposes. At the same time, radiation can also cause damage to normal tissue around the tumor, which has an effect of tumor radiotherapy [2, 3]. Radiation-induced damage of normal tissues restricts the therapeutic doses of ionizing radiation that can be delivered to tumors and thereby limits the effectiveness of radiotherapy [4].

miRNAs are a class of endogenous conserved short non-coding RNAs that regulate gene expression by fully or partially binding to their target gene mRNA sequences [5, 6]. miRNAs are involved in the regulation of almost all biological processes, and increasing evidence indicates that miRNAs play an important role in regulating tumor radiosensitivity [7, 8]. For example, overexpressing miR-182 reduces BRCA1 protein, impairs homologous recombination-mediated repair, and renders cells hypersensitive to IR [9]. Changes in expression of miR-34a and miR-7 may be involved in important protective mechanisms counteracting radiation cytotoxicity [10]. miR-24-mediated down-regulation of H2AX suppresses DNA repair in terminally-differentiated blood cells and renders cells hypersensitive to irradiation and genotoxic drugs [11]. ATM mediates the effect of miR-421 on cell cycle checkpoint and radiosensitivity [11]. Repression of ATR pathway by miR-185 enhances radiation-induced apoptosis and proliferation inhibition [7]. These reports implicate that miRNAs have an important role in DNA damage responses and signaling pathways.

CD47 as an integrin-associated molecule integrin-associated protein was discovered originally as a plasma membrane molecule [13]. CD47 is expressed on the surface of all human solid tumor cells and has been implicated in many tumor pathophysiologic processes includ-

ing tumor cell apoptosis, survival and proliferation, migration, adhesion, and spreading [14]. A major mechanism by which cancer cells evade the innate immune system is by expression of CD47, which is a "don't eat me" signal for phagocytic cells [15]. Recently, CD47 has also been regarded as an attractive radio-therapeutic target because blocking CD47 signaling protects normal tissues while sensitizing tumors to ionizing radiation [16]. However, the mechanism of CD47 involvement in radiobiology and miRNAs that interact with CD47 has not been reported so far.

In the current study, we set out to analyze upregulated miR-222 in response to ionizing radiation. Then, targetScan predicted that the 3' UTR of CD47 was potentially targeted by miR-222. Based upon these findings, we confirmed CD47 as a target of miR-222, and demonstrated that overexpression of the miR-222 enhances cell radiosensitivity by modulating the CD47-pERK pathway in cancer cells.

Materials and methods

Cells and cell culture

For 2D-grown cultures, human cervical carcinoma cell line (Hela), human kidney carcinoma cell line (786-0) and human alveolar adenocarcinoma cell line (A549) were propagated in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 100 U/ml penicillin, and 100 U/ml streptomycin (Amresco, USA). For 3D-grown cultures, the surface of dish was covered with medium-matrigel mixture (volume ratio 1:1) and incubated for 30 min at 37°C to allow the mixture to gel. Then, trypsinized 2D-cultured cells and matrigel were mixed at a volume ratio of 1:1 to 0.5×10^6 cells/ml. The mixture was pipetted onto the pre-coated dish surface and incubated at 37°C to allow them to gel. All cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. In the study, 3D cultured cells were only used in miRNA microarrays, and other experiments used 2D cultured cells as research materials.

Radiation

X-ray irradiation was carried out by a Faxitron RX-650 facility (Faxitron Bioptics, USA), which was operated at 50 kVp 5 mA at room temperature. The target of this instrument is tungsten (W). The dose rate was 0.751 Gy/min.

miRNA microarray

2D cultured and 3D Hela cells were collected after 4 Gy X-ray irradiation. Total RNAs were isolated using TRIzol Reagent (Invitrogen, USA). Then, Cyanine3-pCp was labeled at the 3' end of the RNA and hybridized using Agilent miRNA Complete Labeling and Hyb Kit (Agilent, China). Microarray images were acquired using a Agilent Chip Scanner (G2565CA) and analyzed with Agilent Feature Extraction (v10.7), Agilent GeneSpring and GeneSpring software.

Cell transfection

Based on the sequence of hsa-miR-222 (Accession number: MIMAT0000279), a miRNA mimic was designed and synthesized by Genepharma (Shanghai, China). Double-stranded scrambled RNA (sequence: UUC UCC GAA CGU GUC ACG UTT) was used as the negative control (NC). siRNA that targets CD47 (ID: 145977) and its negative control (AM4611) were purchased from Invitrogen (USA). Prior to transfection, cells were seeded into each well and grown for 24 h. When the cell confluence reached 30-50%, the medium was discarded and replaced in Opti-MEM serum-free medium, and the cells were transfected with oligo-nucleotides using Lipofectamine™ 2000 Reagent (Invitrogen, USA) according to the manufacturer's protocol. After 4-6 h, the medium was changed to normal RPMI-1640 medium containing 10% serum and placed in 37°C in a 5% CO_2 incubator.

Colony formation assay

For clonogenic survival assays, the transfected cells were trypsinized and collected and resuspended in RPMI-1640 medium. Cells were planted in a 60 mm dish at a density of 100-2000 cell/well, depending on the radiation dose. The cells were irradiated at dose rate of 0.749 Gy/min using Faxitron RX-650 facility (Faxitron Bioptics, USA). After irradiation, the cells were immediately incubated in fresh RPMI-1640 media. After incubating for 7-14 days, the cell colonies were formed, and then fixed and stained with 0.5% crystal violet for 20 min. Clusters containing > 50 cells were counted as a colony.

Dual-luciferase reporter assay

A CD47-3'-UTR fragment containing the predicted target site of miR-222 and a CD47-3'-UTR fragment with a mutated target site of miR-222 were chemically synthesized from Sangon Biotech (Shanghai, China). These fragments were annealed and inserted into the pmirGLO Vector (Promega). 786-0 cells were then co-transfected with 200 ng reporter vector and 50 nM miR-222 mimics by using Lipofectamine 2000 in a 96-well plate (Corning). The activities of firefly and renilla luciferase in cell lysates were assayed by using Dual-Glo Luciferase Assay System (Promega) at 24 h post-transfection, and the firefly luciferase activity was normalized to the renilla luciferase activity.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. Primers for mature miR-222 were purchased from GeneCopoeia (Guangzhou, China). U6 was used as an internal control to normalize RNA input, and U6 primers (F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3') were synthesized by Sangon Biotech (Shanghai, China). The primers of CD47 and internal control GAPDH were purchased from GeneCopoeia. The expression of mature miR-222 was quantified by All-in-OneTM miRNA qRT-PCR Detection Kit (Genecopoeia, China). The transcript levels of other genes were quantified by RT^2 Profiler PCR Arrays Kit (Qiagen, German) according to the manufacturer's instructions. Samples were performed by using Bio-Rad Chromo 4 System Real-Time PCR detector (Bio-Rad), and the delta-delta Ct method was used to calculate the fold change.

Western blots

Cells were lysed in RIPA (Beyotime, Shanghai, China) buffer with Protease Inhibitor Cocktail Tablets (Roche) for 10 min on ice. Proteins were separated by 10% SDS-PAGE and transferred to a methanol-activated PVDF membrane (GE Healthcare, Piscataway, NJ, USA). The membrane was blocked for 1 h in PBST containing 5% milk and subsequently probed with anti-CD47 antibody (Santa Cruz Biotechnologies, USA), anti-ERK antibody (Santa Cruz Biotechnologies), anti-p-ERK antibody (Cell Signaling Technology, USA), anti-AKT antibody (Abcam, UK), anti-pAKT antibody (Abcam, UK) and anti-GAPDH antibody (Santa Cruz Biotechnologies) for 2 h. The goat-anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnologies) was incubated for 1 h at room temperature.

Results

miR-222 expression is upregulated in response to ionizing radiation

In order to investigate miRNAs that might be involved in the regulation of cancer cell radiation sensitivity, 2D cultured and 3D cultured Hela cells were treated with 4 Gy X-rays and cells were harvested at 30 min. The miRNA expression profiles were obtained by miRNA microarray analysis. Among the 543 miRNAs checked, miR-222 was one of the miRNAs that were dramatically upregulated both in 2D-cultured and 3D-cultured Hela cells, indicating that miR-222 expression is regulated by ionizing radiation (**Figure 1A**). After irradiation treatment, the expression of Hsa-miR-222 increased by 1.72 times in 2D cultured cells and 9.34 times in 3D cultured cells. Increased expression of miR-222 in 3D cultured cells was more significant than in 2D cultured cells (**Figure 1B**). Increased miR-222 expression was also observed at different radiation doses except for 48 h after treatment (**Figure 1C**).

Elevation of miR-222 sensitizes cancer cells to X-rays

To determine the impact of miR-222 on the radiation sensitivity of tumor cells, Hela and A549 cells were transfected with exogenously synthesized miR-222 mimics at 50 nM and 100 nM, respectively, to elevate miR-222 levels, and then detected the expression of miR-222 by quantitative PCR. The results showed that the expression of miR-222 in Hela and A549 cancer cells increased significantly after transfection with miR-222 mimics (**Figure 2A** and **2B**). A colony formation assay was used to test cell viability of both transfected cells after 4-Gy X-ray irradiation. The survival fractions of Hela and A549 cells transfected with different concentration of miR-222 mimics were significantly lower than those transfected with NC (negative control of miR-222 mimics), indicating reduced cell survival with upregulation of miR-222

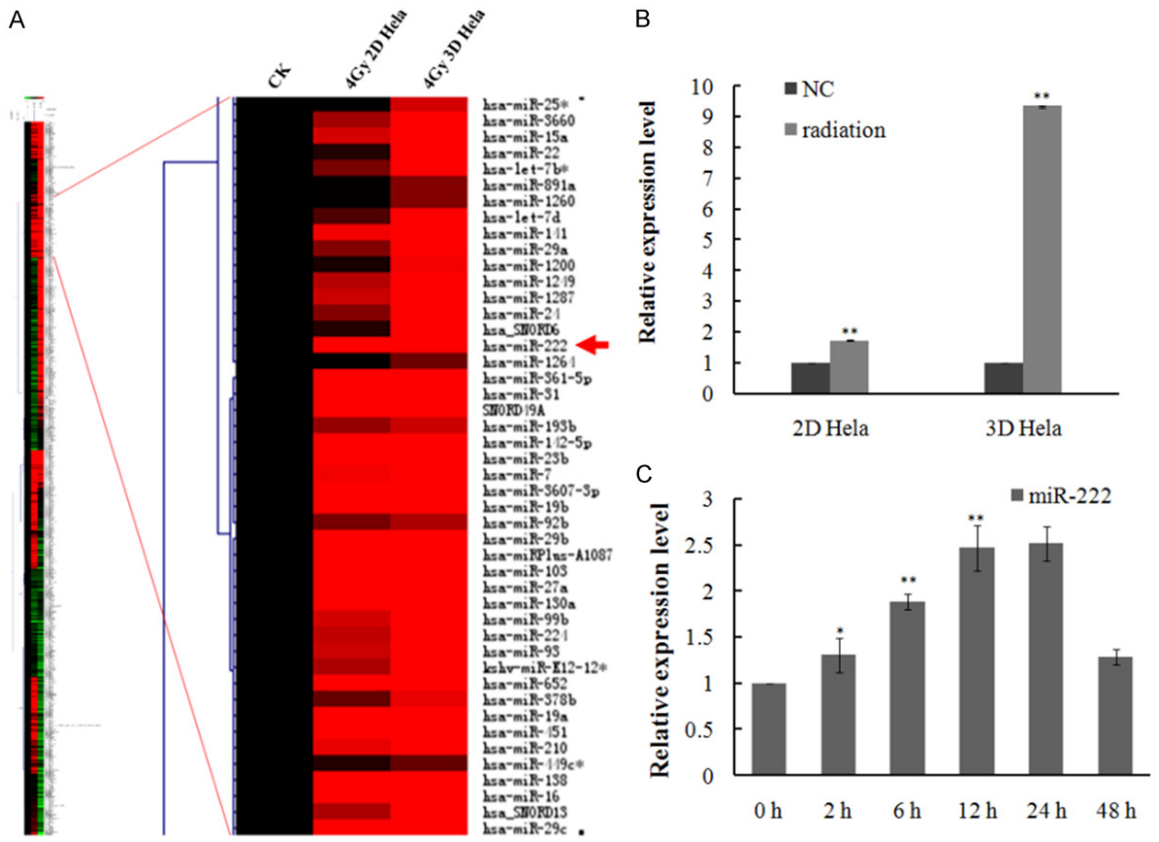

Figure 1. miR-222 expression is upregulated in response to ionizing radiation. A. Hierarchical clustering of miRNA expression obtained with Agilent miRNA microarrays after exposure of 2D-cultured and 3D-cultured Hela cells to 4 Gy of X-rays, respectively. Colored bars represent the differential levels of miRNAs expressed in irradiated samples versus sham-irradiated samples (0 Gy). The red arrow indicates hsa-miR-222. B. Relative changes of miR-222 expression level in 2D-cultured and 3D-cultured Hela cells 30 min after 4 Gy X-ray irradiation, which was also obtained with a miRNA microarray assay. C. Relative changes of miR-222 expression level in Hela cells 0, 2, 6, 12, 24, 48 h after X-ray irradiation. Data with error bars represent the means of at least three independent experiments while others represent the means of two independent experiments. *P < 0.05 compared with the sham-irradiated samples (0 Gy); **P < 0.01 compared with the sham-irradiated samples (0 Gy).

expression (**Figure 2C** and **2D**). To investigate further, we manipulated miR-222 levels in Hela and A549 cells with 50 nM miR-222 mimics and tested the survival fraction after subjecting the samples to 0 Gy, 1 Gy, 2 Gy, 4 Gy of X-rays. As expected, the survival fraction of cells transfected with miR-222 mimics was the lowest among all treatments (**Figure 2E** and **2F**).

miR-222 target CD47

Although p27 Kip1, PTEN, PUMA, Frizzled-7, and PPP2R2A have been reported to be targets of miR-222 [17-21], it is unclear whether any target of miR-222 is involved in the radiation response. To address this issue, we predicted the target gene of miR-222 by combining several predictive tools available online, including Microrna Targets Version 5 (http://www.microrna.org) and Targetscan detection (http://www.targetscan.org). Among the predicted targets that showed significant matches with the miR-222 sequence, we focused on CD47 because it is regarded as an attractive radiotherapeutic target. Above all, there are no reports describing CD47 regulation by miRNA up to date. One highly conserved putative binding site was found in the CD47 3'-untranslated region (3'-UTR) (**Figure 3A**).

To validate the target prediction, we constructed the vector by inserting either the wild-type sequence of the 3'-UTR of CD47 mRNA (CD47-3'-UTR) or a mutated seed sequence of the miR-222-binding site (CD47-3'-UTR-mut) into the pMIR-REPORT luciferase reporter. Our

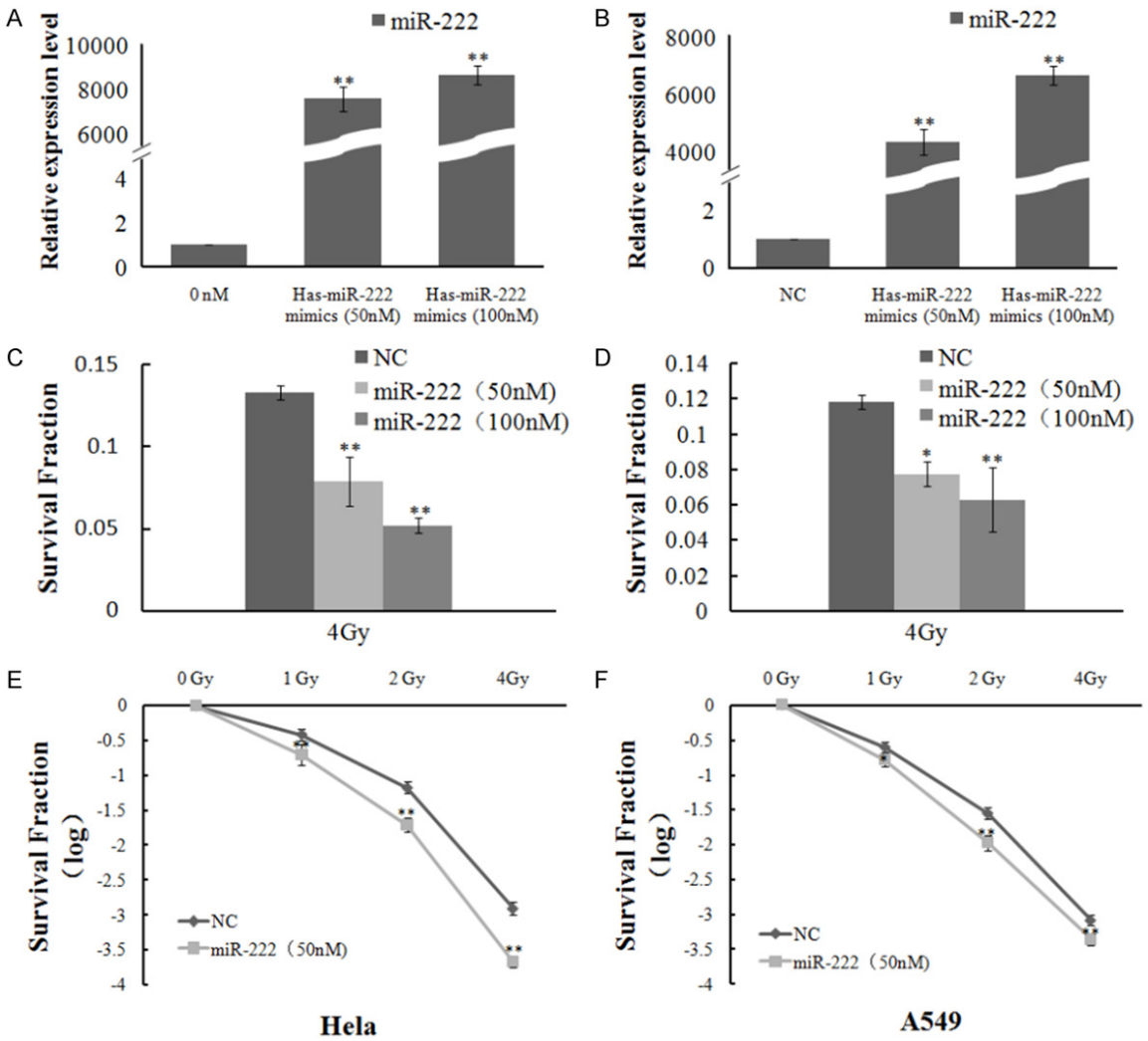

Figure 2. Impact of miR-222 levels on cell survival. A, B. Detection of miR-222 expression level after transfection of Hela and A549 cells with different concentrations of miR-222 mimics (50 and 100 nM final). C, D. Survival fractions of Hela and A549 cells exposed to 0 and 4 Gy of X-rays after transfection with NC and miR-222 mimics (50 nM final). E, F. Survival fractions of Hela and A549 cells with NC and cells transfected with miR-222 mimics (50 nM final) in response to 0, 1, 2 and 4 Gy of X-rays measured by colony formation assay. Each experiment was conducted at least three times independently. *P < 0.05 compared with NC treatment; **P < 0.01 compared with NC.

results show that co-transfection of the vector with wild-type CD47-3'-UTR and miR-222 mimics inhibited luciferase activity whereas co-transfection of the vector with CD47-3'-UTR-mut and miR-222 mimics caused no inhibition of luciferase activity in 786-0 cells (**Figure 3B**). Subsequently, the qRT-PCR experiments showed that CD47 mRNA decreased 24 h after Hela cells were transfected with miR-222 (**Figure 3C** and **3D**). Western blot showed that the expression of CD47 decreased after Hela cells were transfected with miR-222 mimics (**Figure 3E**). These results indicate that miR-222 directly regulates the expression of CD47 by targeting the 3'-UTR of its mRNA.

Knockdown of CD47 enhances radiation sensitivity of tumor cells

Having established a negative regulatory link between miR-222 and CD47, we then focused on CD47 implications in radiobiologic effects. Hela cells were exposed to X-rays and then measured the expression of mature CD47 by quantitative qRT-PCR and western blot. As shown in **Figure 4A**, the expression of CD47 gradually decreased from 2 hours to 24 hours,

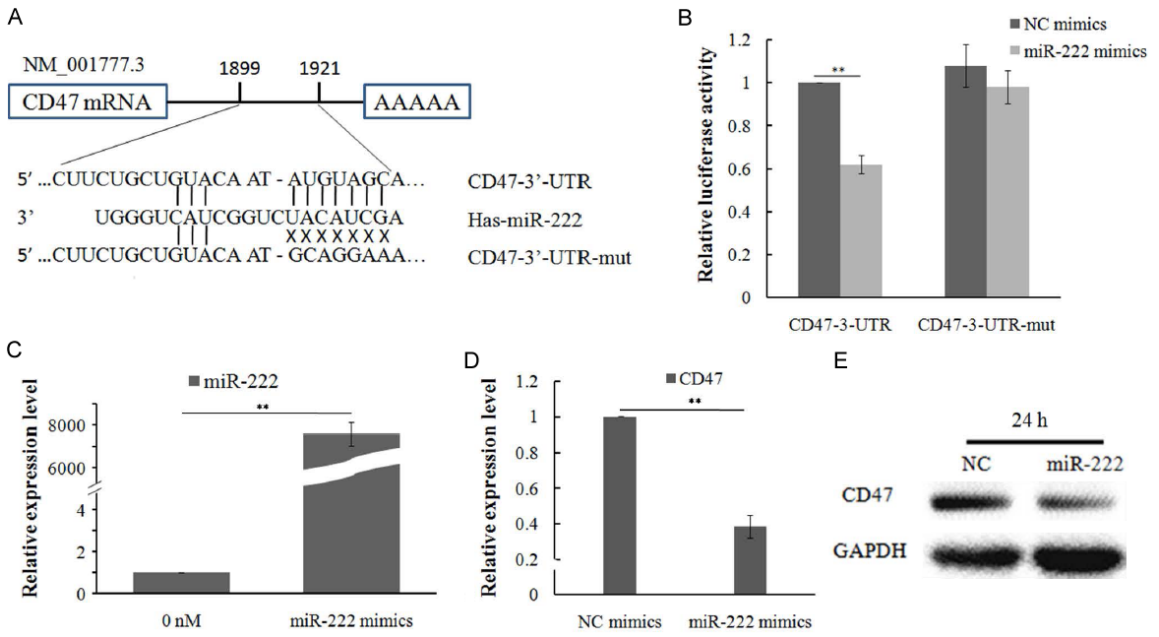

Figure 3. MiR-222 negatively regulates CD47 expression at the post-transcriptional level. A. Putative miR-222 binding sites within the human CD47 3'-UTR are shown at the top. Sequences of mature miR-222 aligned to target site and the UTR mutated in the miR-222 seed-pairing sequence are shown below. B. Luciferase reporter assays were performed following co-transfection in 786-O cells with WtCD47 or Mut CD47 vectors together with miR-222 mimics or nonsense small RNA oligonucleotides as the negative control (NC). Luciferase activity was read 24 h after transfection. C. qRT-PCR assay of miR-222 mRNA expression at 24 h after being transfected with miR-222 mimics or NC into 786-O cells. D. qRT-PCR was conducted to quantify the expression level of CD47 mRNA at 24 h after 786-O cells were transfected with miR-222 mimics or NC. E. Western blot assay of CD47 protein level at the indicated time point after being transfected with miR-222 or NC into 786-O cells. Significance was determined by Student's t-test. *, $P < 0.05$; **, $P < 0.01$.

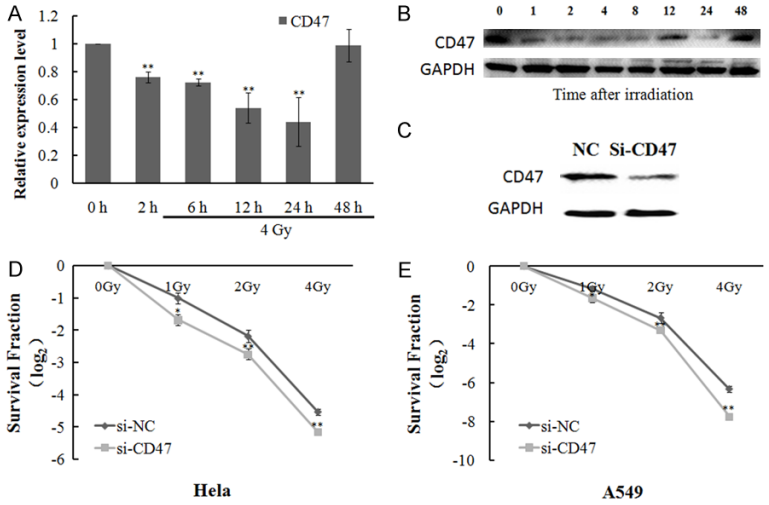

Figure 4. Response of CD47 to ionizing radiation. A. Expression levels of CD47 mRNA were measured by qRT-PCR at the indicated time points after irradiation with 4 Gy X-rays. GAPDH was used as internal control. B. Western blot assay of CD47 protein level at the indicated time point after irradiation with 4 Gy X-rays. C. CD47 protein levels were measured by western blot in Hela cells after transfection with CD47-siRNA or negative control (NC). D, E. The survival fractions of Hela and A549 cells transfected with siRNA-CD47 with negative control (si-NC), respectively. Significance was determined by Student's t-test. *, $P < 0.05$; **, $P < 0.01$.

and its expression increased to the level before radiation treatment after 48 hours (**Figure 4A**). The CD47 protein levels were further confirmed by western blot. Consistent with previous study [22], the CD47 protein level was significantly changed in Hela cells until 1 h after being exposed to 4 Gy X-rays. The protein started to increase at the 12 h time point and reached a relatively high level at 48 h (**Figure 4B**).

To further validate whether CD47 sensitizes Hela and A549 cells to X-ray radiation, we knocked down CD47 by using siRNA in Hela and A549 cells and then observed whether this also induces the same radiobiologic effects as

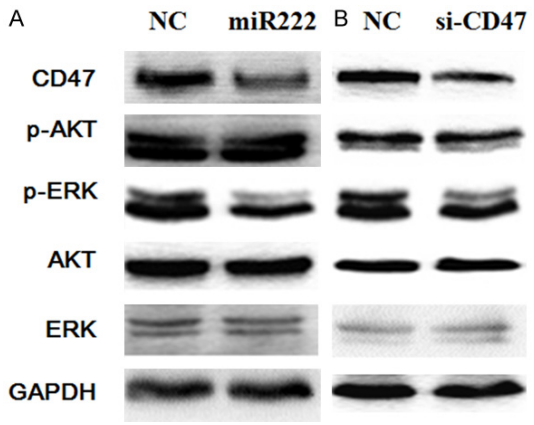

Figure 5. miR-222 regulates the radiation sensitivity of cancer cells by repressing the p-ERK pathway. A, B. Western blotting was performed to measure the protein levels of CD47 p-AKT, p-ERK, AKT, and ERK in cells transfected with miR-222, CD47-siRNA with transfection (Ctrl) 24 h, respectively.

those of the overexpression of miR-222. The survival fractions of Hela and A549 cells transfected with siRNA-CD47 were lower than those transfected with negative control as shown in **Figure 4D** and **4E**, suggesting that the knockdown of the CD47 increases the radiosensitivity of Hela and A549 cells.

miR-222 enhances radiosensitivity of cancer cells by repressing p-ERK pathway

Knowing the negative regulatory relationship between miR-222 and CD47, we were then interested in the mechanisms of sensitization of miR-222 in Hela cells. It have been reported that CD47 plays a role in tumor growth by regulating the ERK and AKT pathway [16, 23]. First, we confirmed the induction of p-ERK pathway after transfection of miR-222 mimics into Hela cells. The protein levels of p-ERK were decreased in the transfected samples when compared with the NC transfection, while the expression of p-AKT protein was not changed significantly (**Figure 5A**). Consistent with miR-222 mimic-transfected cells, the p-ERK protein levels was also declined in CD47 siRNA transfected Hela cells compared to NC (**Figure 5B**). ERK pathway is involved in radiation response of cancer cells has been reported [23, 24]. Above all, these results demonstrate that miR-222 regulate the radiation sensitivity of cancer cell by repressing the p-ERK pathway, which can be induced by intrinsic and extrinsic cellular stresses, and results in increased sensitivity of cancer cells.

Discussion

Irradiation is part of the therapeutic plan for more than half of cancer patients [1]. Normal tissue damage caused by radiation is a major side effect of tumor radiation therapy. Although precise field and accurate dosing schedules are used to limit this damage to adjacent tissues, it still occurs in most patients and ultimately limits the effectiveness of radiotherapy [4].

CD47 is a signaling receptor for thrombospondin-1 and has been found to be expressed in multiple human tumor types, while it is ubiquitously expressed at low levels in normal cells [25, 26]. Multiple tumors express increased levels of CD47 compared with their normal cell counterparts. Thismakes CD47 a potential target for cancer immunotherapy [27, 28]. CD47 has been proved to be involved in radiation biological effects. CD47 blockade confers a survival advantage to irradiated normal tissue in an autophagy-dependent manner [29]. Inhibition of CD47 signaling maintains the viability of normal tissues after irradiation while increasing the radiosensitivity of tumors [16]. However, little was known about the regulatory mechanisms for CD47 expression in response to radiation. Moreover, the targets of miR-222 that are tightly linked to radiation responses remain unclear. Here, we demonstrate that miR-222 targets CD47 directly by binding to the 3'-UTR of CD47 mRNA. CD47 expression is downregulated by miR-222 at both mRNA and protein levels. Our results also show that miR-222 enhances Hela and A549 cell radiosensitivity through repressing of CD47. To the best of our knowledge, this is the first report on the regulation of CD47 expression mediated by a miRNA.

miR-222 was identified as a key miRNA, located on human chromosome Xp11.3 as a single transcript, and showing high sequence identity [30]. Deregulation of some miRNAs, including miR-222, has been observed in lymphoma, colorectal, lung, and breast cancers, glioblastoma, and gastric cancer [31-34]. It has been reported that upregulation of miR-221/miR-222 significantly decreased radiosensitivity of gastric carcinoma SGC7901 cells by repress-

ing PTEN [35]. Here, we observed upregulation of miR222 expression in Hela cells after X-ray irradiation. Subsequently, overexpression of miR222 enhances the radiosensitivity of tumor cells Hela and A549. This is the opposite of the above results, suggesting that miR-222 may regulate other PTEN-independent signaling pathways to enhance Hela and A549 cells' radiosensitivity. Supporting this view is that the functional roles of miR-222 are variable, acting as oncogenes or tumor suppressors, depending on the tumor system [30]. Although p27Kip1, PUMA, PTEN, TIMP3, SODI, MMP1, and ETS1 have been identified as targets of miR-222 [18, 36-40], we further confirmed that CD47 is also a target gene of miR222, and that miR-222 enhances the radiosensitivity of Hela and A549 cells by inhibiting CD47. It is well known that the relationship between miRNA and target mRNA is not a "one-to-one" connection, and that the choice of how many and which miRNAs target one 3'-UTR is strongly determined by the particular cellular environment [18, 36-40]. Thus, the same miRNA exerts different, or even opposite, effects in different tumors, depending primarily on which targets drive tumor cell behavior in that particular cellular environment [41].

AKT and ERK pathways are involved in the response to radiation. It has been demonstrated that X-ray treatment induces A375 cell growth inhibition by triggering reactive oxygen species-mediated DNA damage involving inactivation of AKT and MAPKs [42]. Shen *et al.* found that ERK signaling contributes most significantly in radioresistant cells [24]. Furthermore, oncogenic activation of ERK plays an important role in the CD47 signaling pathway [23]. CD47 promotes human glioblastoma invasion through activation of the PI3K/Akt pathway [28], and activation of CD47 receptors causes proliferation of human astrocytoma but not normal astrocytes by an AKT-dependent pathway [43], suggesting miR-222 enhances radiosensitivity of Hela and A549 by repressing CD47 may be mediated through ERK and AKT pathway. To investigate the mechanism of miR-222 in regulating cancer cell radiosensitivity, western blot was used to detect changes in protein levels of AKT and ERK in Hela cells after transfection with miR222 mimics and CD47-siRNA. The results showed that p-ERK level decreased and p-ERK remained unchanged relative to NC transfected cells, demonstrating that miR-222 represses the expression of CD47. It inactivates its downstream effector p-ERK. We favor a model in which elevation of miR-222 enhances cancer cell radiosensitivity by downregulating the CD47-pERK pathway (**Figure 5**).

Taken together, we demonstrated that miR-222 could regulate cancer cell radiosensitivity by targeting CD47. Our results provide strong evidence that miR-222 has an important biologic effect on cancer cell radiosensitivity. Elucidation of this mechanism offers opportunities for application of miR-222 in therapeutic intervention and radiosensitization. However, it is noteworthy that the results in this study are based on only in vitro cultured Hela and A549 cell lines that might not necessarily comprehensively reflect the in vivo situation. Therefore, further experiments are required to elucidate the mechanisms of miR-222 in in vivo systems.

Acknowledgements

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Disclosure of conflict of interest

None.

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