# MicroRNA-320 regulates the radiosensitivity of cervical cancer cells C33AR by targeting β-catenin

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**Abstract.** Cervical cancer is the second most common malignancy in women worldwide and always has recurrence owing to radioresistance. MicroRNA (miRNA or miR) has been identified to relate to the sensitivity of cancer radiotherapy. Here, we investigated the potential of miRNA-320 as a biomarker for radiosensitivity by targeting β-catenin in cervical cancer. A radioresistant cervical cancer cell line, C33AR, was established, and the radioresistance of C33AR cells was confirmed by a colony-formation assay. The expression of miRNA-320 was detected by reverse transcription-quantitative polymerase chain reaction, and compared between C33A and C33AR. β-catenin, the target of miRNA-320, was determined at the protein level by western blotting after transfecting the inhibitor of miRNA-320. The expression of miRNA-320 was markedly decreased in C33AR cells, which appeared to be more radioresistant, compared with its parental cell line C33A. Target prediction suggested that miRNA-320 negatively regulated the expression of β-catenin. Knockdown of β-catenin increased C33AR radiosensitivity, which revealed that the inhibition of β-catenin could rescue the miRNA-320-mediated cell radioresistance. On the other hand, overexpressing miRNA-320 increased C33AR radiosensitivity. In conclusion, miRNA-320 regulated the radiosensitivity of C33AR cells by targeting β-catenin. This finding provides evidence that miRNA-320 may be a potential biomarker of radiosensitivity in cervical cancer.

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## Introduction

Cervical cancer, the second most common malignancy in women worldwide, is often diagnosed at a local and advanced stage (1). Radiotherapy is one of the most important treatments (1). However, radioresistance (inherent and acquired) often circumvents the efficacy of radiotherapy (2). Many patients have tumour recurrence due to radiotherapeutic resistance (2). However, the mechanisms of radioresistance are poorly understood.

MicroRNAs (miRNAs or miRs) are a class of non-coding RNAs, ~20-22-nucleotides long, which negatively regulate the expression of many cancer-related genes by binding to 3'-untranslated regions (UTRs) (3-5). Considerable evidence suggests that miRNAs are associated with multiple processes, including the tumorigenesis, proliferation and migration of many types of cancer (6-9). For example, miRNA-610 downregulated vasodilator-stimulated phosphoprotein to influence the invasion and migration of gastric cancer cells (10), and miRNA-144 promoted cell proliferation, migration and invasion in nasopharyngeal carcinoma through repression of phosphatase and tensin homolog (11). Growing evidence suggests that some miRNAs are related to radioresistance, such as miRNA-31, miRNA-181, miRNA-324-3p and miRNA-214 (12-15). However, the functions of miRNAs in radioresistance are still largely unknown. In this study, X-rays induced the expression of miRNA-320 in the radioresistant cervix cancer subline C33AR. Furthermore, target prediction suggested that miRNA-320 influences cervical cancer radiosensitivity by targeting β-catenin. The activation of β-catenin plays a crucial role in human cancers through the canonical Wnt/β-catenin signaling pathway, and the expression of β-catenin in cervical carcinoma causes a malignant phenotype (16). Our findings therefore suggest that a decrease in the expression of miRNA-320 promotes radioresistance in the C33AR cervical cancer cell line by permitting β-catenin expression.

## Materials and methods

*Cell culture and transfection.* The human cervical cell line C33A was obtained from the Shanghai Life Science

Institute Cell Library (Shanghai, China). C33A and the acquired radioresistant cell line C33AR were cultured in Minimum Essential Medium (MEM) (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), penicillin-streptomycin liquid (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 0.25 $\mu$g/ml amphotericin B (Amresco, Inc., Framingham, MA, USA) in a humidified atmosphere of 5% $CO_2$ at 37˚C. The miRNA-320 agomir (a novel class of chemically engineered miRNA mimic) and negative control were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells were transfected with 100 nM miRNA-320 agomir or negative control using Lipofectamine 2000 in Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A miRNA-320 inhibitor antiagomir (Guangzhou RiboBio Co., Ltd.) was designed to suppress the expression of miRNA-320. Small interference RNA (siRNA) was used to inhibit β-catenin expression. The siRNAs against β-catenin as well as the non-targeting control siRNAs were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were transfected with siRNAs at a concentration of 25 nM using Lipofectamine 2000. The sequence of the β-catenin siRNA was 5'-GGACACAGCAGCAAUUUGUTT-3'.

*Establishing a radioresistant cervical cancer cell line*. Cells were cultured to 80% confluence and then subjected to irradiation. Irradiation parameters were set as follows: Quality, 6 MV/X-rays; dose rate, 2 Gy/min; field, 35x35 cm; and 2 Gy each time, using a linear accelerator. Cells were kept at room temperature for ≤30 min during irradiation. One set of flasks was not irradiated and treated as wild type. After 24 h of incubation, the medium in each flask was exchanged for fresh medium to remove detached cells. Cells resistant to radiation were cultured in the same medium, which was exchanged for fresh medium every 2 days thereafter. To generate stable radioresistant clones, all the clones after 60 Gy radiation were expanded for >6 months without radiation to confirm the radioresistant phenotype before studies were undertaken. Two clones from C33A cells were established. The parental cells of wild-type stable clones were generated under the same conditions without irradiation.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and analysis*. Total RNA was extracted from cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For the detection of miRNAs, RNA was reverse transcribed using a specific RT primer for U6 and miRNA-320 (Guangzhou RiboBio Co., Ltd.) according to the protocol of the manufacturer. qPCR was carried out with SYBR Green I Mix (Takara Biotechnology Co., Ltd., Dalian, China) in a 20-$\mu$l reaction volume (12.5 $\mu$l SYBR Green I Mix, 0.5 $\mu$l ROX reference dye II, 200 mM forward and reverse primer, 2 $\mu$l complementary DNA template and 8 $\mu$l double-distilled $H_2O$) on the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the following protocol: 95˚C for 20 sec, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 70˚C for 10 sec. The primers for miR-212/320/132/15a/16 for RT-qPCR were designed and synthesized by Guangzhou RiboBio Co., Ltd. The sequences for the remaining primers were as follows: β-catenin forward, 5'-GAAACGGCTTTCAGTTGAGC-3' and reverse, 5'-CTGGCCATATCCACCAGAGT-3'; c-MYC forward, 5'-GGGTAGTGGAAAACCAGCAGC-3' and reverse, 5'-CCTCCTCGTCGCAGTAGAAATA-3'; cyclin D1 forward, 5'-GAGGAACAGAAGTGCGAGGAG-3' and reverse, 5'-GGATGGAGTTGTCGGTGTAGAT-3'; and GAPDH forward, 5'-TGGAAGGACTCATGACCACA-3' and reverse, 5'-TTCAGCTCAGGGATGACCTT-3'. Δ quantification cycle (Cq) was calculated by subtracting the Cq of U6 from the Cq of miRNA-320. ΔΔCq was then calculated by subtracting the ΔCq of the control from the ΔCq of the treatment group. The fold-change of miRNA expression was calculated by the equation $2^{-\Delta\Delta Cq}$ (10).

*Flow cytometric analysis of cell cycle distribution*. Cells were harvested and washed with cold PBS three times, fixed with 70% ethanol at 4˚C for 24 h, washed again three times with cold PBS and then stained with 50 mg/ml propidium iodide for 0.5 h at 37˚C (Beyotime Institute of Biotechnology, Haimen, China). DNA content was analyzed on the Cytomics FC 500 (Beckman Coulter, Inc., Brea, CA, USA).

*Colony-formation assay*. Cells in an exponential growth phase were plated into a 6-well plate at 100, 200, 400, 800, 1,000 and 2,000 cells per well, and then irradiated with 0, 2, 4, 6, 8 and 10 Gy. Cells were then incubated at 37˚C in 5% $CO_2$, 95% air for 9-12 days. When most cell clones had reached >50 cells, they were stained with 0.5% (w/v) crystal violet (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). The number of clones in the radioresistant group was compared with that in the wild-type group. The survival curves were obtained and analyzed with GraphPad Prism 4 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). Three independent experiments were performed.

*Cell proliferation assay*. Cell proliferation was assessed using the WST-1 Cell Proliferation Reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. The C33AR cells were plated into a 96-well cell culture plate at 2,000 cells/well and then incubated at 37˚C overnight to allow settling. Then, they were transfected with miRNA-320 agomir and negative control, and treated with 4 Gy X-ray radiation. Subsequently, 10 $\mu$l of WST-1 reagent was added in each well and incubated for 2 h at 37˚C. Absorbance was subsequently determined at a wavelength of 450 nm (for measurements) and 650 nm (as reference) by a microplate reader (EnSpire; PerkinElmer, Inc., Waltham, MA, USA). Cell proliferation was calculated by subtracting the absorbance values of the samples from that of the medium alone (background level). The relative cell proliferation was normalized to that of the control group.

*β-catenin RNA interference in the cervical cancer radioresistant cell line C33AR*. The β-catenin siRNA oligonucleotides with the sequence si-1 sense 5'-GCAGUUGUAAACUUGAUUATT-3'; si-2 sense 5'-CCCAAGCUUUAGUAAAUAUTT-3'; and si-3 sense 5'-GGACACAGCAGCAAUUUGUTT-3', along with the corresponding antisense

oligonucleotides, were synthesized by Shanghai GenePharma Co., Ltd. A negative control siRNA (Shanghai GenePharma Co., Ltd.) was used as a control siRNA. siRNA transfection was performed using Lipofectamine 2000 as indicated in the manufacturer's instructions. Briefly, subconfluent C33AR cells were plated in 6-well plates in regular growth medium. The next day, they were transfected with either 100 nM control siRNA or the β-catenin siRNAs for 6 h, followed by recovery in serum-containing medium. After 48 h of siRNAs transfection, the cells were harvested for protein isolation analysis.

*Western blotting*. Cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM $Na_3VO_4$, 1 mM EDTA, 1 mM EGTA and a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Cell lysates were quantified for protein content by the bicinchoninic acid (BCA) method using a BCA kit (Bio-Rad Laboratories, Inc.). Then, 40 $\mu$g of protein was resolved in 12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked in 5% bovine serum albumin (Beyotime Institute of Biotechnology) in TBS with Tween-20 (TBST) for 2 h, and then probed with primary antibodies against β-catenin (ab22656; Abcam, Cambridge, MA, USA), cyclin D1 (ab134175; Abcam), c-MYC (ab32072; Abcam) and GAPDH (KM1002T; Sungene Biotech Co., Ltd., Tianjin, China) at a 1:1,000 dilution at 4˚C overnight, washed extensively with TBST three times, and incubated with secondary antibodies conjugated with horseradish peroxidase (Pierce; Thermo Fisher Scientific, Inc.)at room temperature for 2 h at a 1:5,000 dilution. Immunoreactive protein was examined using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology).

*Statistical analysis*. The results of the quantitative data in this study are expressed as the mean ± standard deviation. The Student's *t*-test was used to evaluate the significant difference between two groups of data in all pertinent experiments with the statistical package SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ (using a two-tailed paired *t*-test) was considered to indicate a statistically significant difference between two groups of data.

## Results

*Establishing and validating radioresistant C33AR cells*. To generate a radioresistant cell line, C33A cells in exponential growth phase were exposed to X-rays at a dose of 2 Gy and a dose rate of 2 Gy/min. An interval of 1 to 3 weeks between each ionizing radiation (IR) allowed the surviving cells to regenerate. The whole process of IR and culture lasted for ~1 year, and the surviving cell line was termed C33AR. To verify phenotypes, C33AR cells were irradiated and examined by the colony-formation assay. Compared with C33A, C33AR showed no change in foci formation when IR was absent, but gained more foci and higher survival fractions when exposed to IR (Fig. 1A), establishing C33AR as a stable radioresistant cell line. Next, flow cytometry was used to evaluate alterations in the cell cycle (Fig. 1B and C). After irradiation, C33AR cells in the G1 and S phases significantly increased. Conversely, the proportion of C33AR in the G2/M phase decreased.

*Differential expression of miRNAs and β-catenin in radioresistant cells*. The differential miRNA expression profile between C33AR cells and their parental C33A cells was determined using RT-qPCR (Fig. 2A). The expression of miRNA-320 in C33AR cells was significantly lower than that in C33A cells. Several differentially expressed miRNAs in this profile were previously reported to have a role in tumorigenesis, including the development of radioresistance (17,18). These results indicated that differentially expressed miRNAs may contribute to the acquisition of radioresistance in cervical cancer. The expression of miRNA-320 was significantly changed in cervical cancer cells after radiation. In addition, β-catenin was highly expressed in C33AR cells compared with C33A cells at the protein level but not at the messenger RNA level (Fig. 2B and C).

*miRNA-320 influences the sensitivity of C33AR and parental cells to irradiation*. Based on the differential expression of miRNA-320 between C33AR and its parental C33A cells, the potential role of miRNA-320 in cervical cancer radiobiology was examined by overexpressing or repressing miRNA-320 using synthetic miRNA-320 agomir/antiagomir in C33AR and C33A cells, respectively. The expression of miRNA-320 was tested by RT-qPCR (Fig. 3A and B). Following miRNA-320 overexpression, the survival rate of C33AR cells decreased compared with that of C33AR cells transfected with negative control vector 10 days after IR stimulation (Fig. 3C). These results revealed that overexpression of miRNA-320 significantly increases the sensitivity of C33AR cells to irradiation. The expression of miRNA-320 in C33A cells was inhibited by being transfected with antiagomir, and caused a decrease in radiosensitivity (Fig. 3D). In the proliferation assay, C33AR cells were transfected with miRNA-320 agomir and negative control, and exposed to IR with 4 Gy x-rays, and their cell growth was monitored by counting the cell numbers (Fig. 3E). Increased expression of miRNA-320 promoted cell death in the experimental group with 4 Gy IR exposure in the long-term cell culture compared with the negative control.

*Decrease in miRNA-320 induces the radioresistance of cervical cancer cells by targeting β-catenin expression*. Hsieh *et al* have reported that miRNA-320 inhibits endogenous β-catenin expression and nuclear localization in prostate cancer (19). When β-catenin translocates to the nucleus, it activates the transcription of Wnt/β-catenin target genes, such as c-MYC and cyclin D1 (20). Therefore, to further explore the mechanism by which miRNA-320 influences cell radiosensitivity, the expression of β-catenin, c-MYC and cyclin D1 was examined by RT-qPCR and western blotting after transfecting C33AR cells with miRNA-320 agomir (Fig. 4A, B and D). Upon transfection with miRNA-320 antiagomir in C33A cells, the expression of β-catenin increased at the protein level (Fig. 4C). To confirm that miRNA-320 modulates the radiobiological behavior of cervical cancer cells by repressing β-catenin expression, rescue experiments were performed. First, β-catenin expression was inhibited using siRNAs (Fig. 4E) and then, the knockdown of β-catenin

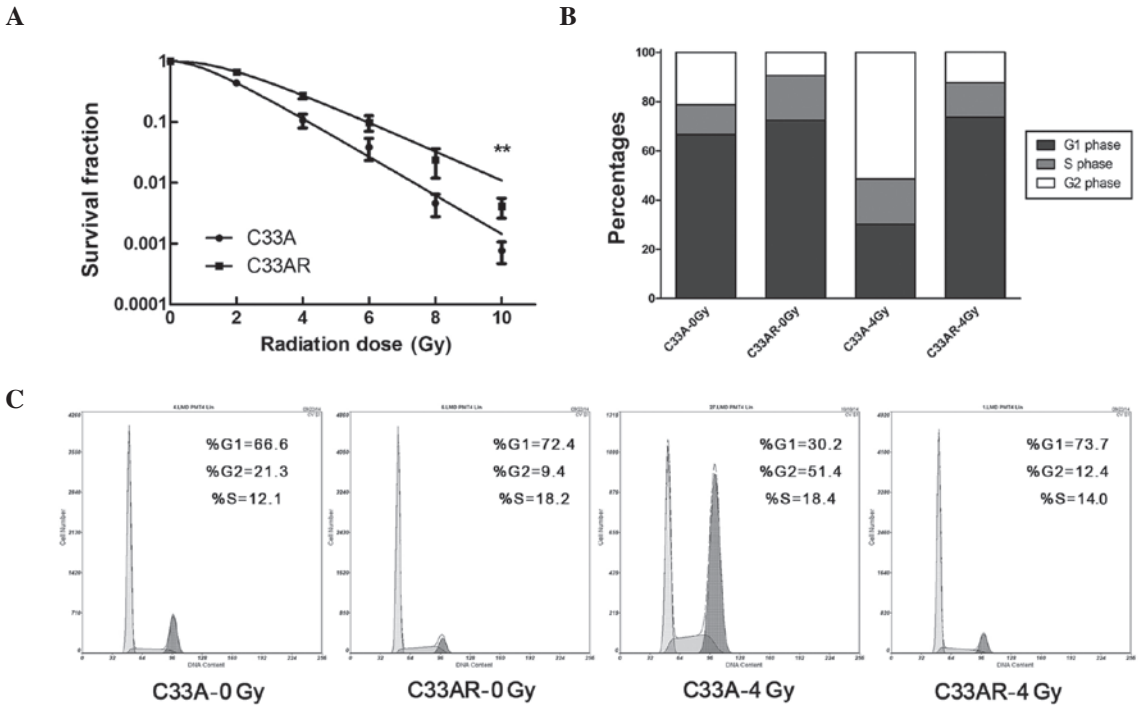


Figure 1. Establishing and validating radioresistant C33AR cells. The acquired radioresistant cell line C33AR was examined for its radiobiological characteristics. (A) Survival curves of C33A and C33AR cells. Cell surviving fractions were normalized to the plating efficiency of non-irradiated cells. **P<0.05. (B) The percentage of cells in each cell cycle phase of C33A and C33AR cells following 4 Gy X-ray radiation. (C) Cell cycle distribution of C33A and C33AR cells after 4 Gy X-ray radiation. Error bars represent SD. Means ± SD are representative of three independent experiments. SD, standard deviation.

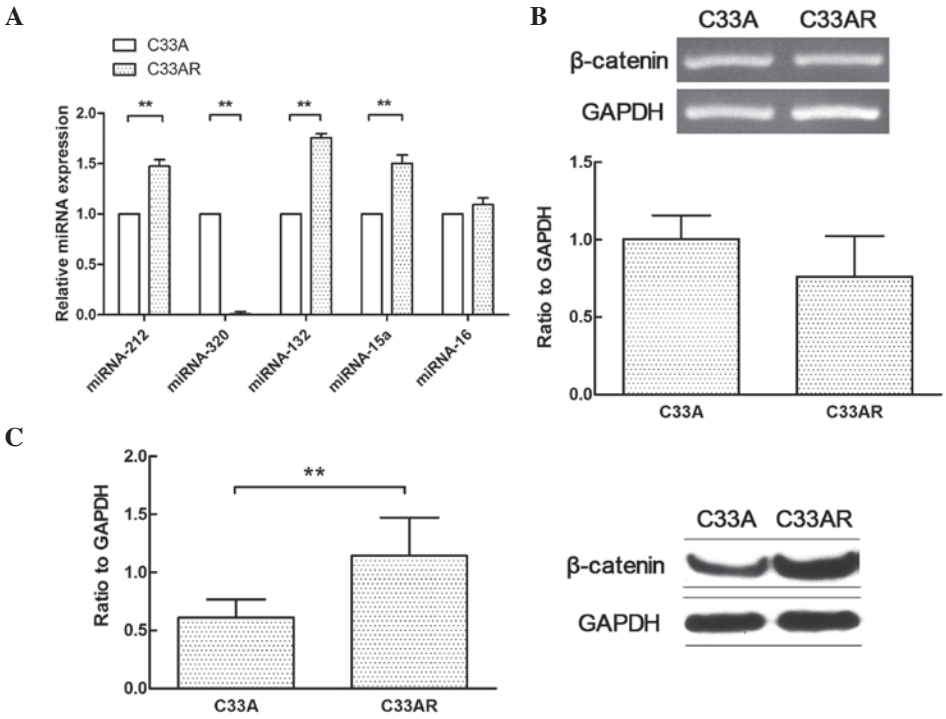


Figure 2. Differential expression of miRNAs and β-catenin in radioresistant cells. Expression profiles of miRNAs and β-catenin in C33A and C33AR cervical cell lines. (A) The expression of miRNAs, as analyzed by reverse transcription-quantitative polymerase chain reaction and relative to U6, was different between parental cells C33A and radioresistant cells C33AR. (B) β-catenin mRNA expression was measured using GAPDH as an internal control, and no significant difference was observed between C33A and C33AR. (C) The expression of β-catenin protein was assessed by western blotting, and was observed to be increased in C33AR compared with C33A. The data were expressed as means ± standard deviation (**P<0.05, Student's *t*-test). miRNA, microRNA.

increased C33AR radiosensitivity (Fig. 4F). The inhibition of β-catenin rescued miRNA-320-mediated cell radioresistance. These results indicated that a decrease in miRNA-320 inhibits cervical cancer cell radiosensitivity *in vitro* by negatively regulating β-catenin expression and Wnt/β-catenin signaling activity.

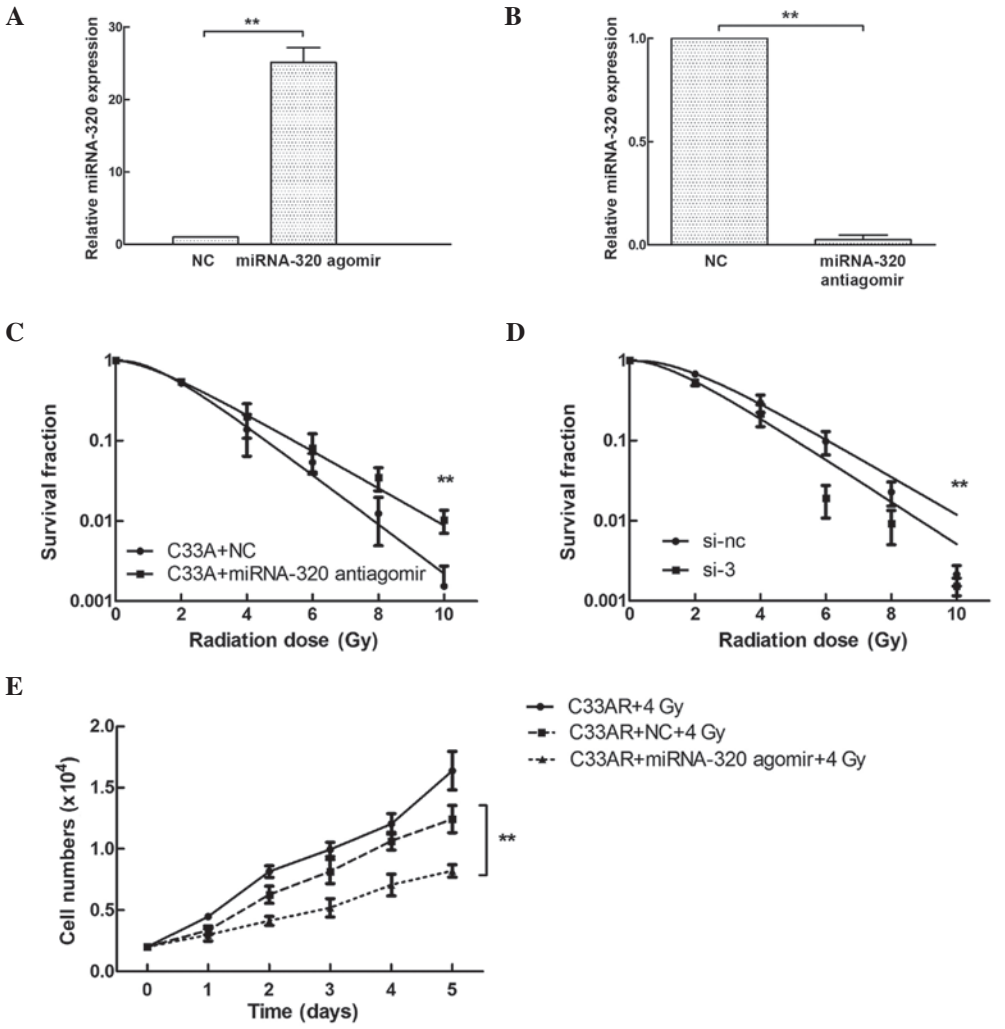


Figure 3. miRNA-320 influences the sensitivity of C33AR and parental cells to irradiation. Overexpression or repression of miRNA-320 in C33AR and its parental C33A influences the sensitivity of the cells to irradiation. (A and B) The expression of miRNA-320 was examined by reverse transcription-quantitative polymerase chain reaction after transfection of miRNA-320 agomir/antiagomir in (A) C33AR and (B) C33A cells. (C) miRNA-320 overexpression in C33AR cells results in increased radiosensitivity. (D) The expression of miRNA-320 was inhibited in C33A cells and caused a decrease in radiosensitivity. Quantitative measurement of colony formation in each cell group. (E) Proliferation of C33AR cells after transfection with a miRNA-320 agomir and then exposure to ionizing radiation with 4 Gy. The results were the mean of three independent experiments ± standard deviation ($^{**}P<0.05$). miRNA, microRNA; NC, negative control; si, small interfering RNA.

## Discussion

Radiotherapy is the main treatment for cervical cancer, especially at an early stage of the disease. However, many patients are not sufficiently radiosensitive and relapse soon after radiotherapy (1). The tumor radiation response is thus a major factor for the effect (and closely related to) tumor radiosensitivity. Numerous biological processes participate in the regulation of tumor radiation response, including DNA damage response and repair, cell cycle checkpoint, apoptosis control, and metabolism (21).

Although some achievements have been made in previous studies (22,23), the exact molecular mechanisms underlying radioresistance remain to be elucidated. In the past years, there has been increasing evidence regarding miRNAs as important regulators of radiotherapeutic resistance and other biological effects (24,25). In this study, a radioresistant cervical cancer cell line was identified using the clonogenic assay. Biological analyses revealed an increased percentage of G1-phase cells in the C33AR cell line compared with the parental cell line, suggesting its radioresistance, since it is generally accepted that cells are usually most sensitive to radiation in the late G2/M phase and most resistant to radiation in the mid-to-late S and early G1 phases (26).

The RT-qPCR results indicated that miRNA-320 was significantly decreased in the C33AR cell line. The *in vitro* functional analysis in our study demonstrated that a decrease in miRNA-320 confers radioresistance to the C33AR cell line, and that miRNA-320 overexpression induces increased radiosensitivity in C33AR cells. This alteration suggests that the differential expression of some miRNAs may be important for surviving the cytotoxic effects of radiation, thus supporting previous results demonstrated in other cancer types (27,28).

miRNA-320 is located on chromosome 8p21.3, a region frequently reported to undergo a loss of heterozygosity during the progression of prostate cancer (29). Altered miRNA-320 expression has also been linked to a defect in post-transcriptional processing and chromosomal

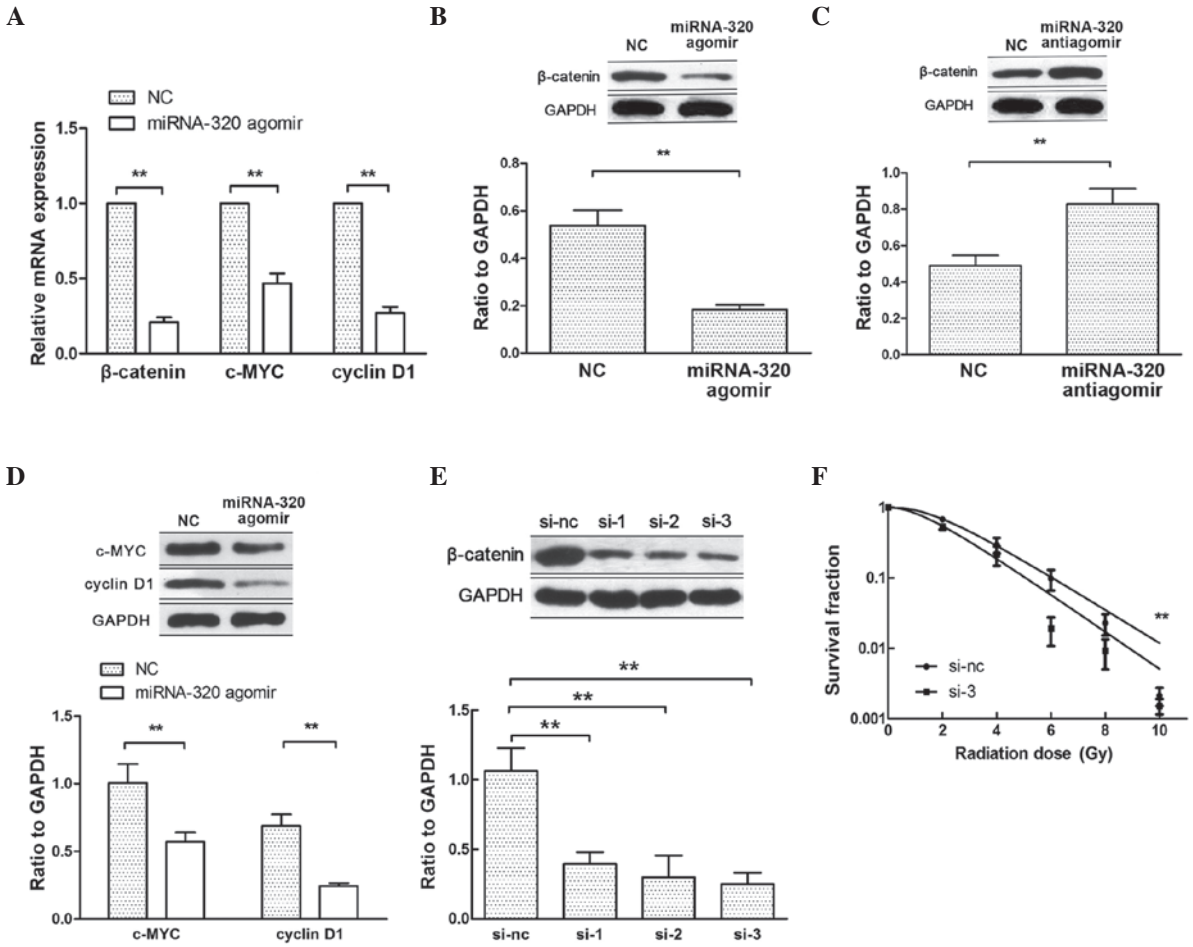


Figure 4. Decrease in miRNA-320 induces the radioresistance of cervical cancer cells by targeting β-catenin expression. Determination of β-catenin as a target of miRNA-320 and effect of β-catenin on the radiosensitivity of cervical cancer cells. (A) The mRNA expression of β-catenin, c-MYC and cyclin D1 was decreased after transfection with miRNA-320 agomir. (B) Decreased expression of miRNA-320 facilitates the expression of β-catenin. (C) Increased expression of miRNA-320 inhibits the expression of β-catenin. (D) The protein level of β-catenin, c-MYC and cyclin D1 was decreased after transfection with miRNA-320 agomir. (E) siRNA-mediated inhibition of β-catenin in C33AR cells. (F) β-catenin inhibition rescues miRNA-320-mediated radioresistance of C33AR *in vitro*. C33AR cells were transfected with si-3 and si-NC, and the survival fractions were determined. The results shown (mean ± standard deviation) are representative of three independent experiments (**P<0.05). miRNA, microRNA; NC, negative control; si/siRNA, small interfering RNA; mRNA, messenger RNA.

deletions (29). The attenuation of miRNA-320 expression has also been demonstrated to be important for the initiation (30) and progression (31) of a number of cellular processes. The downregulation of miRNA-320 and the following radioresistant effect in C33AR cells support a critical role for this miRNA in modulating the cellular response to radiation. This is supported not only by the downregulation of miRNA-320 in our model but also in the larynx squamous carcinoma acquired radioresistant cell line Hep2R (32) in a previous study. However, the overexpression of miRNA-320 in the C33A cell line did not enhance radiosensitivity (data not shown). This may be explained by a plateau effect, given the relatively high basal expression of miRNA-320 in these cells, or it may suggest that the modulatory effect of miRNA-320 on the radiosensitivity of C33AR cells may depend on other molecular genetic changes that occurred during the generation of this radioresistant subline.

Radiation can induce different alterations in various oncogenes or cancer suppressor genes, including P53, Bcl-2-associated X protein, P21 and DNA-dependent protein kinase (33-35). Therefore, bioinformatic algorithms, including PicTar (pictar.bio.nyu.edu/), MicroRNA.org (www.microrna.org/microrna/home.do) and Targetscan (www.targetscan.org/), were used to determine that β-catenin, a well-known oncogene (36), is a potential target with the highest predictive value for miRNA-320. In addition, a previous study confirmed it as a target of miRNA-320 (19). The study, performed in prostate cancer cells, demonstrated that miRNA-320 influences stem cell-like characteristics by directly downregulating the Wnt/β-catenin signaling pathway (19). In the present study, miRNA-320 was identified as a critical contributor to radioresistance by directly targeting β-catenin in cervical cancer. Previous studies have demonstrated that the activation of the Wnt signaling pathway is a key radioprotective mechanism in irradiated cancer cells (37-40). Woodward *et al* reported that Wnt and β-catenin signaling may contribute to the radioresistance of breast cancer stem cells (41), and Watson *et al* reported that cells with silenced β-catenin are more sensitive to radiation compared with the parental cell line (42). The present report is the first to demonstrate an association between miRNA-320 downregulation and radioresistance through a negative

regulation of β-catenin. Although the colony-formation assay confirmed that the differential expression of miRNA-320 and the inverse expression of β-catenin are related to radioresistance, it remains unclear how the miRNA-320/β-catenin signaling pathway participates in establishing radioresistance in cervical cancer. Further work is required to elucidate these factors and to assess their importance in the radiation response of cervical cancer.

As traditional radiotherapy may result in the potential radioresistance of cervical cancer, the classical schedule requires a radiosensitive drug regimen to enhance its curative effect. The roles of miRNAs in the regulation of tumor radiosensitivity suggest that miRNAs will be a promising target for clinical diagnosis and treatment. In addition, the potential improvement of radiotherapeutic effects through activating or inhibiting the expression of certain miRNAs and downstream target genes is extremely promising. A thorough understanding of tumor radiosensitivity and the regulatory mechanisms of miRNAs will bring new hope to more cancer patients.

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