Review Article
MicroRNA-129-5p-mediated inhibition of autophagy enhanced the radiosensitivity of human colon cancer cells

Jiayou Xu, Fuchang Zheng, Wensheng Yu

Department of General Surgery, Weifang People’s Hospital, Weifang, Shandong, China

Received December 4, 2015; Accepted September 29, 2016; Epub December 1, 2016; Published December 15, 2016

Abstract: The efficacy and outcome of radiotherapy in the treatment of colon cancer are much limited by radioresistance. Studies show microRNAs are involved in radioresistance in many cancers. In the present study, we aimed to explore the role of microRNA-129-5p in regulating radioresistance of colon cancer cells and its underlying mechanism. In the present study, we established a radioresistant colon cancer cell line. Compared with control group, miRNA-129-5p was significantly reduced whereas the autophagy was enhanced in radioresistant colon cancer cell line. Exotic expression of miRNA-129-5p was capable to inhibit beclin-1, a critical autophagy gene, and suppressed autophagy activity. Furthermore, we found beclin-1 was the direct target of miR-129-5p as evidenced by in silico analysis and luciferase reporter assay. In addition, overexpression of miRNA-129-5p inhibited cell growth and colony formation ability and promoted irradiation-induced apoptosis of radioresistant colon cancer cells. By contrast, overexpression of beclin-1 abolished the effects of miRNA-129-5p. In vivo, miRNA-129-5p sensitized xenograft tumor to irradiation. Taken together, the present study suggested that miRNA-129-5p significantly augmented the radiosensitivity of colon cancer cells through inhibiting beclin-1-mediated autophagy, hinting a promising new molecular target for the treatment of colon cancer.

Keywords: microRNA-129-5P, autophagy, beclin-1, radiosensitivity, colon cancer

Introduction
Malignant colon cancers are the most common primary adult tumor and one of the most difficult tumors to treat [1, 2]. Nowadays preoperative chemoradiation therapy has become an integral component in the treatment for local advanced rectal cancer, which is believed to be able to reduce the risk of local recurrence and increase the probability of sphincter-preserving surgery [3]. However, the radioresistance of colon cancer cells severely limits the efficacy and outcomes of radiation therapy in clinical treatment [4, 5]. For example, it is estimated that only about 20% of patients achieve complete pathologic responses to preoperative radiation therapy [6]. Thus, it is urgently needed to overcome radioresistance of colon cancer cells.

MicroRNAs (miRNAs) are a type of highly conserved non-coding small RNAs which post-transcriptionally regulate the expression of their target genes [7, 8]. miRNAs bind to 3’-untranslated region (UTR) of the target mRNA leading to mRNA destabilization and thereby inhibition of protein translation. Recently, the role of miRNAs in tumorigenesis and cancer treatment has been intensively studied [9-11]. miRNAs are associated with patient survival and are useful predictors and modifiers for anticancer therapy [12, 13]. However, the potential underlying mechanism of miRNAs in regulating radioresistance of colon cancer cells remains unknown.

Autophagy is a cellular process that is responsible for the degradation of cytoplasmic proteins and organelles [14, 15]. Autophagy is a membrane trafficking process which involves the autophagosome formation [16]. Beclin-1 is a key gene for autophagosome formation that exhibits high levels during autophagy [17]. In addition, conversion of microtubule-associated protein light chain 3 (LC3) from LC3-I to LC3-II is
another critical process during autophagy and therefore the ratio of LC3I/II has been widely accepted as a classic marker for the assessment of autophagy activity [18]. In most circumstances, autophagy acts as an adaptive response to cell stress such as nutrient starvation or metabolic stress [19, 20]. Recently, studies show that autophagy is also critical for tumor cellular response to survive stressful conditions, and thus has been implicated in the radiosensitivity of tumor cells [21-23]. Thus, targeting autophagy to enhance the radiosensitivity of colon cancer cells is a promising research direction for improving clinical efficiency and outcome of radiotherapy.

In the present study, we explored the role of miRNA-129-5p in radioresistance of colon cancer cells and the underlying mechanism of its effects from autophagy-related perspective.

Materials and methods

Cell culture and animal preparation

The human colon cancer cell line, HT-29, was cultured in DMEM (Invitrogen-Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen-Life Technologies, Carlsbad, CA, USA) containing penicillin/streptomycin. The cells were grown in a humidified 5% CO₂ at 37°C in an incubator. The irradiation of HT-29 cells was performed according to a previously reported method [24]. HT-29 cells cultured in complete medium were subjected to 2 Gy ⁶⁰Co radiation at 2 Gy/min using an X-ray machine (X-RAD 320, PRECISION X-ray). The irradiated cells were then cultured in new plates and irradiated with increasing doses of irradiation (4, 6, 8 and 10 Gy) for subsequent experiment.

BALB/c female nude mice, 6-week-old, were bought from the Experimental Animal Center of Shandong University (Shandong, China) and housed under pathogen-free conditions with free access to water and food. The animal experimental procedures were approved and reviewed by the Institutional Animal Care and Use Committee of Shandong Medical University.

Cell viability and colony formation assays

After irradiation and/or miR-129-5p treatment, cell growth and viability were assessed by MTT assay kit (Sangon, Shanghai, China). Briefly, the cells were seeded in 96-well plates at a density of 5 × 10³ cells/200 µl. 10 µl of 5 mg/ml MTT labeling reagent were added to HT-29 cells, and the plate was incubated for 4 h in a humidified incubator at 37°C. After the incubation, the absorbance of the samples was measured at a wavelength of 570 nm with 655 nm as a reference wavelength. For detection of the colony formation ability, the cells following treatment were grown in 6-well plates and cultured for 15 days. The old medium was discarded and cell colonies stained with crystal violet were counted using microscope. The experiments were performed in triplicates.

TUNEL assay

Apoptotic cells was detected using a TUNEL kit (Sigma, St. Louis, MO, USA) according to the manufacturer’s instructions and previous study [25]. Briefly, cells were cultured on cover slips for 48 h. Then the cells were fixed in 4% paraformaldehyde solution in PBS for 30 min at room temperature. The cells were then incubated with a methanol solution containing 0.3% H₂O₂ for 30 min at room temperature to block endogenous peroxidase activity, and then incubated in the TUNEL reaction mixture for 60 min at 37°C, and visualized by fluorescence microscopy (DM4000B; Leica Wetzlar, Germany). Apoptotic cells were counted from four randomly selected fields in each sample.

Dual-luciferase reporter assay

The beclin-1 3’-UTR and mutated 3’-UTR constructs were amplified and sub cloned into pGL3 Luciferase Promote Vector (Sangon, Shanghai, China) with XbaI and NotI restriction sites. Using Lipofectamine transfection reagent (Sangon, Shanghai, China) the pGL3 vector containing beclin-1 3’-UTR or mutated forms was co-transfected with or without miR-129-5p mimic (GenePharma, Shanghai, China) into HT-29 cells according to the manufacturer’s instruction. The cells were collected after 48 h transfection and the luciferase activity was measured using the Dual-Luciferase Reporter Assay Kit (Sangon, Shanghai, China). The relative protein expression levels of beclin-1 and LC3-II were quantified using Image-Pro Plus 6.0 software. The relative luciferase activities were normalized with GAPDH to that of the control cells.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated by using TRIzol (Invitrogen-Life Technologies, Carlsbad, CA, USA)
MicroRNA-129-5p-autophagy pathway in colon cancer

and small RNAs were extracted by using mirVana kits (Invitrogen-Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Corresponding cDNA was generated by using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA) and the TaqMan miRNA Reverse Transcription Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer’s instructions. To analyze the gene expression levels, the RT-qPCR mixture system containing cDNA templates, primers and SYBR-Green qPCR Master Mix were subjected to RT-qPCR quantification. GAPDH for beclin-1 and U6 SnRNA for miR-129-5p were used as an internal reference.

Western blot analysis

Briefly, the proteins were electrophoresed by sodium dodecyl sulfate polyacrylamide gel (Bio-Rad) and transferred to polyvinylidene fluoride membranes (Millipore, MA). The membranes were blocked with 5% skimmed milk in Tris-buffered saline-Tween (TBST) and then incubated with a primary antibody with gentle shake at 4°C for 12 h. Further, the membranes were washed three times with TBST and incubated with a peroxidase-conjugated secondary antibody. Primary antibodies including anti-LC3-I and anti-LC3-II and anti-beclin-1 antibodies (Abcam, Cambridge, UK), anti-cleaved caspase-3 (Sigma, St. Louis, MO, USA) were used. GAPDH (Sigma, St. Louis, MO, USA) was used as an internal control. The target protein was visualized by an enhanced chemiluminescence (ECL) detection system (Invitrogen-Life Technologies, Carlsbad, CA, USA). All values were calculated as ratios normalized to GAPDH.

**Tumorigenicity assay**

Cells (5 × 10^7) diluted in 200 µl PBS were injected subcutaneously into the right groin of BALB/c nude mice. The tumor volume was measured daily and the tumor was irradiated with a dose of irradiation (10 Gy) when the volume reached about 500 mm^3_. The length and width were measured and the volume was calculated using the formula: length × width^2 × π/6.

**Statistical analysis**

Data were presented as mean ± SD from at least three independent experiments. Data between or among groups were analyzed by two-tailed Student’s t-test or one-way ANOVA by SPSS 19.0 software (SPSS Inc., Chicago, IL, USA), respectively. P<0.05 was considered to be statistically significant.

**Results**

**Irradiation inhibited miRNA-129-5p and activated autophagy in HT-29 cells**

In order to explore the potential role of miRNA-129-5p in radioresistance of colon cancer...
cells, we established a radioresistant HT-29 cell line and examined miRNA-129-5p level in radioresistant cells by qRT-PCR. Compared with the control HT-29 cells, miRNA-129-5p was significantly downregulated in radioresistant (RR)-HT-29 cells (Figure 1A). Western blot analysis showed an increased autophagy activity as indicated by upregulation of beclin-1 and LC3-II protein in RR-HT-29 cells as compared with control cells (Figure 1B-D). The above data demonstrated that irradiation significantly inhibited miRNA-129-5p and activated autophagy.

**Exotic expression of miRNA-129-5p decreased autophagy activity**

To explore the potential relationship between the decreased miRNA-129-5p and the increased autophagy in RR-HT-29 cells, we transfected RR-HT-29 cells with miRNA-129-5p mimics and determine the alteration of autophagy. Western blot showed that miRNA-129-5p significantly inhibited beclin-1 as well as LC3-II protein levels, suggesting that miRNA-129-5p played an important role in the regulation of autophagy activity (Figure 2A-C).

**Beclin-1 is a direct target of miR-129-5p**

We used TargetScan on the internet to determine the relationship between miRNA-129-5p and beclin-1. As expected, we found that beclin-1 was one of the potential targets of miR-129-5p. The predicted binding of miR-200b with beclin-1 3′UTR is illustrated (Figure 3A). To validate interaction between miR-129-5p and beclin-1, the beclin-1 complementary sites, with or without mutations, were cloned into the 3′UTR of the firefly luciferase gene and co-transfected with miR-200b mimics or scramble
miRNA in HepG2 cells. Our results showed that, the presence of miR-129-5p lead to a significant reduction in the relative luciferase activity of the wild-type construct of beclin-1 3'UTR in HT-29 cells. However, the mutant (MUT) construct of beclin-1 3'UTR abolished such the suppressive effect of miR-129-5p in HT-29 cells (Figure 3B). The above results demonstrated...
that beclin-1 is the direct target of miR-129-5p.

**Exotic expression of miR-129-5p inhibited cell proliferation and colony formation in RR-HT-29 cells in response to irradiation**

We unregulated the miR-129-5p in RR-HT-29 cells and assessed the growth and colony formation of RR-HT-29 cells in response to irradiation. RR-HT-29 cells were pretreated with miR-129-5p mimics or control scramble miRNA for 1 h and then exposed to irradiation (2 Gy/min). MTT assay showed that, compared with the control group, miR-129-5p overexpression significantly sensitized RR-HT-29 cells to irradiation-induced cell death (Figure 4A). In addition, miR-129-5p markedly inhibited the colony formation ability of RR-HT-29 cells (Figure 4B). These data suggested that miR-129-5p sensitized RR-HT-29 cells to irradiation.

**miR-129-5p enhanced the radiosensitivity of RR-HT-29 cells via activation of cell apoptosis**

Furthermore, we determined the effects of miR-129-5p on cell apoptosis in RR-HT-29 cells in response to irradiation. TUNEL assay showed that miR-129-5p upregulation significantly increased the cell apoptosis of RR-HT-29 cells as compared with the control (Figure 4C and 4D). Besides, cleaved caspase-3, the pro-apoptotic protein, was also increased by miR-129-5p (Figure 4E and 4F). The above results dem-

---

**Figure 5.** Effect of up-regulated beclin-1 on miR-129-5p-induced irradiation sensitivity in RR-HT-29 cells. (A) RR-HT-29 cells were treated with miR-129-5p with or without beclin-1 overexpression vector were subjected to irradiation (2 Gy/min) and cultured for 48 h. The levels of beclin-1 were determined by western blot. Cells treated with scramble oligonucleotides were regarded as control. (B) Relative protein expression level of beclin-1 was measured using Image J and normalized to β-actin. Cell proliferation and apoptosis was detected by MTT assay (C) and TUNEL assay (D) respectively. *P<0.05 and **P<0.01 compared with the indicated group.
MicroRNA-129-5p-autophagy pathway in colon cancer

Figure 6. Effect of miR-129-5p on xenograft tumor growth in vivo. A. Nude mice were subcutaneously injected with HT-29 cells infected with control lentivirus vector or miR-129-5p overexpression lentivirus vector. The tumors were irradiated (10 Gy) on day 15 when the average tumor volume reached about 500 mm³. Tumor volume was monitored over time as indicated. B. The tumors were excised on day 40. miR-129-5p overexpression caused a significant decrease in tumor volume.

Overexpression of beclin-1 abolished miR-129-5p-enhanced radiosensitivity of RR-HT-29 cells

In order to determine whether miR-129-5p enhanced radiosensitivity of RR-HT-29 cells via regulating beclin-1, we co-transfected miR-129-5p with beclin-1 overexpression vectors into RR-HT-29 cells. MTT assay showed that beclin-1 overexpression significantly blocked miR-129-5p-induced cell growth inhibition in RR-HT-29 cells in response to irradiation (Figure 5A-C). Furthermore, beclin-1 overexpression abolished cell apoptosis induced by miR-129-5p in RR-HT-29 cells as evidenced by TUNEL assay (Figure 5D). The above results suggested that miR-129-5p targeted beclin-1 to sensitize RR-HT-29 cells to irradiation.

miRNA-129-5p sensitized xenograft tumor to irradiation

To determine whether miRNA-129-5p can increase the efficiency of irradiation in killing implanted tumors in nude mice, we subcutaneously injected nude mice with HT-29 cells pre-transfected with lentiviral vector expressing miRNA-129-5p or scramble miRNA control. When the tumors reached about 500 mm³ on day 15, a single dose of 10-Gy irradiation was given. The results showed that miRNA-129-5p significantly increased the radiosensitivity of HT-29-derived tumors on irradiation treatment (Figure 6A and 6B). These in vivo findings confirmed that miRNA-129-5p sensitized colon cancer cells to irradiation.

Discussion

In the present study, we demonstrated that microRNA-129-5p was significantly decreased in radioresistant colon cancer cell line. Overexpression of miRNA-129-5p markedly inhibited cell growth and colony formation and promoted irradiation induced cellular apoptosis. Further, we demonstrated that the action of miRNA-129-5p was through inhibiting beclin-1 mediated autophagy and confirmed that beclin-1 was the direct target of miRNA-129-5p. In addition, Exotic upregulation of beclin-1 significantly abrogated the radiosensitivity-enhancing effect of miRNA129-5p. Finally, in vivo, miRNA-129-5p was shown to sensitize xenograft tumor to irradiation. Taken together, the present study suggested that miRNA-129-5p significantly augmented the radiosensitivity of colon cancer cells through inhibiting beclin-1-mediated autophagy, hinting a promising new molecular target for the treatment of colon cancer.

microRNAs have been characterized as important regulatory mechanisms of gene expression and intensively studied in tumorigenesis and cancer treatment [26, 27]. miR-129-5p was firstly identified to regulate the progression of hepatocellular carcinoma through targeting VCP/p97 [28]. Also, many other studies show that miR-129-5p play important antitumor roles in diffuse large B cell lymphoma, cervical cancer cells and ovarian cancer cell [29-32]. However, research on the role of miR-129-5p in colon cancer cells is still lacking. In the present study, we demonstrated that miR-129-5p significantly enhanced the radiosensitivity of colon cancer cells and robustly inhibits colon cancer cells proliferation and survival. Importantly, we demonstrated the action of miR-129-5p in colon cancer cells was via downregulating beclin-1, suggesting beclin-1 as a potential target in clinical treatment for colon cancer.
Autophagy, an adaptive response against cellular stress, has been currently shown to be involved in the radioresistance of cancer cells [10, 33]. In glioma, radiation induces autophagy, which is responsible for the radioresistance of glioma stem cells [34, 35]. In breast cancer, inhibition of autophagy promoted radiosensitivity via inhibiting transforming growth factor-activated kinase-1 [36]. In addition, hypoxia-induced autophagy was demonstrated to contribute to the radioresistance of breast cancer cells [37, 38]. The role of autophagy in the regulation of radioresistance of pancreatic cancer was also investigated. Profilin1 is capable to sensitize pancreatic cancer cells to irradiation by suppressing autophagy [39]. As expected, the present study demonstrated miR-129-5p was involved in the regulation of radioresistance through inhibiting beclin-1 mediated autophagy. Our study reinforced the evidence for the role of autophagy in regulating radioresistance of cancer cells.

In conclusion, our present study provides evidence that miR-129-5p directly targeted beclin-1 to inhibit autophagy, which significantly enhanced the radiosensitivity of colon cancer cells.

Acknowledgements

Research supported by the Shandong province natural science foundation of China (NO. ZR2009CM116).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jiayou Xu, Department of General Surgery, Weifang People’s Hospital, 151 Guangwen Street, Kuiwen District, Weifang 261041, Shandong, China. E-mail: xujiayou121@163.com

References

MicroRNA-129-5p-autophagy pathway in colon cancer


