FEBS Letters 587 (2013) 436–443

FEBS Letters

journal homepage: www.FEBSLetters.org

Differential roles of miR-199a-5p in radiation-induced autophagy in breast cancer cells

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ARTICLE INFO

Article history:
Received 16 October 2012
Revised 18 December 2012
Accepted 26 December 2012
Available online 18 January 2013

Edited by Tamas Dalmay

Keywords:
MiR-199a-5p
DRAM1
Beclin1
Autophagy
Irradiation

ABSTRACT

Autophagy is a self-degrading process that is triggered by diverse stimuli including ionizing radiation. In this study we show novel phenomena in which transfection of miR-199a-5p mimic significantly suppresses IR-induced autophagy in MCF7 cells, and up-regulates basal and IR-induced autophagy in MDA-MB-231 breast cancer cells. We also identify *DRAM1* and *Beclin1* as novel target genes for miR-199a-5p. Overexpression of miR-199a-5p inhibits DRAM1 and Beclin1 expression in MCF7 cells, while it enhances expression of these genes in MDA-MB-231 cells. Furthermore, we show that miR-199a-5p sensitizes MDA-MB-231 cells to irradiation. Therefore, our data identify miR-199a-5p as a novel and unique regulator of autophagy, which plays an important role in cancer biology and cancer therapy.

1. Introduction

Autophagy is an evolutionarily conserved intracellular catabolic process in which a cell degrades long-lived proteins and damaged organelles, such as the endoplasmic reticulum, Golgi apparatus, and mitochondria [1]. Autophagy is active at basal cellular growth levels to function as endogenous cleaning system, and also can be triggered by diverse stressful conditions, such as adaptation to starvation, oxidative or genotoxic stress, and elimination of pathogens [2,3]. And thus, deregulated autophagy has been linked to pathologic conditions such neurodegenerative diseases, cardiomyopathy, and cancer [4]. The exact role of autophagy in carcinogenesis is still elusive. Autophagy can behave as a tumor suppressor or oncogene. The similar paradox is exhibited during tumor therapy, in which autophagy could play pro-survival role and deteriorate the cancer therapeutic outcome or autophagy could work as programmed cell death to ameliorate the over all anti-tumor efficacy [5]. Therefore, achieving better molecular understanding of autophagy and the discovery of specific autophagy modulators suitable for in vivo use will help to dramatically improve cancer therapy [6]. MicroRNAs (miRNAs), the short (~22 nucleotides) non-coding RNAs, have emerged recently as novel endogenous gene regulators. They bind by incomplimentary base pairing to the 3′-untranslated region (3′UTR) of their target mRNA to post-transcriptionally suppress gene expression [7]. MiRNAs have been shown to play important roles in virtually all fundamental cellular events like cell proliferation and apoptosis [8,9]. MiRNAs were found to be deregulated in various body tumors and affect crucial signaling networks which control carcinogenesis. And hence miRNAs are being categorized as tumor suppressors and oncogenes [10]. MiR-17–92 cluster has been found to be overexpressed and possesses oncogenic potential in human B cell lymphoma [11], lung [12] and colorectal cancer [13]. MiR-let-7 expression was found to be lower in lung tumors than in normal lung tissue, and replacement of miR-let-7 suppressed lung cancer growth via targeting the RAS proto-oncogene [14]. Until very recently, accumulating studies showed that miRNAs are novel autophagy modulators in human cancer cells [15]. MiRNA-30a and miRNA-376b have been demonstrated to target and inhibit Beclin1 and thereby blocking autophagy in cancer cells [16,17]. MiR-199a-5p has been reported to deregulated in several aggressive tumor types [18–22], suggesting that this miRNA could have distinct pathophysiological functions. Down-regulation of miR-199a-5p was observed in hepatocellular [23], breast and testicular cancers [24,25]. Moreover, recent studies indicated that miR-199a-5p is a putative tumor suppressor in human liver and testicular cancer cells [23,25]. Despite all these studies, functions and the target genes

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.12.027

of miR-199a-5p are largely unknown especially in breast cancer and need to be discovered. Due to the importance of autophagy in cancer biology and therapeutics, we were interested to explore the impact of miR-199a-5p on the process of autophagy and identify the related target genes in human breast cancer cells.

2. Materials and methods

2.1. Cell lines and treatment

Human breast cancer cell lines, MCF7 and MDA-MB-231 were cultured at 37 °C in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humid incubator with 5% CO_2. Cells were transfected with 100 nM of miR-199a-5p mimic or Negative Control (NC) (GenePharma, Shanghai, China) using lipofectamine™ 2000 (Invitrogen) followed by IR. NC has a unique sequence designed such that it does not target any human genes. (Mimic and NC sequences are listed in Supplementary Table S1).

2.2. Radiation

180-KVp X-ray generator (Model XSZ-Z20/20, Dandong, liaoning, China) was utilized to deliver radiation at a dose rate of 0.41 Gy/min (200 kV; 18 mA).

2.3. RNA extraction

Total RNA was extracted 48 h after transfection with mimic or NC, using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Samples were stored at −80 °C before use.

2.4. Quantitative real-time (qRT)-PCR detection of miR-199a-5p

miRCURY LNA™ Universal RT microRNA PCR (Exiqon) was used for detection of miRNA expression by quantitative real-time PCR on the Stratagene MX3000p thermocycler according to the manufacture's protocol. 20 ng of RNA was used for reverse transcription and the reverse transcription mixture was incubated at 42 °C for 60 min followed by heat-inactivation of the reverse transcriptase for 5 min at 95 °C. cDNA template was diluted 80-fold in nuclease free water. Melt curve was made to determine the optimal condition. The PCR protocol is as follows: denaturation 95 °C for 10 min, then 40 amplification cycles (95 °C for 10 s and 60 °C for 1 min, at a ramp-rate of 1.6 °C/s). U6 sequence was used as a normalization control for all samples.

2.5. Bioinformatics predict for target gene of miR-199a-5p

MiRNA target genes were predicted by union of miRBase Target v4 (http://www.mirbase.org/), PicTar 4.0 (http://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org/), followed by screening for availability of gene symbols in NCBI human sequences (http://www.ncbi.nlm.nih.gov/).

2.6. Plasmid construction

The 3′-untranslated region (3′UTR) of DRAM1 (GenBank ID: NM_018370.2, region from 2572 to 3543 bp) and BECN1 (Genbank ID: NM_003766, region from 1531 to 2145 bp) carrying putative miR-199a-5p binding site were amplified by PCR from human genomic DNA of healthy blood donor. DRAM1–3′UTR was then cloned in XbaI sites of pGL3-control vector (Promega), and BECN1–3′UTR was cloned in between SacI and MluI sites of pMIR-REPORT™ luciferase vector (Ambion). PCR with appropriate primers also generated inserts with mutated miR-199a-5p complementary sites. All PCR products cloned into the plasmid were verified by DNA sequencing to ensure that they were free of mutations and in the correct cloning direction. (Primer sequences are listed in Supplementary Table S1.)

2.7. Luciferase reporter assays

MCF7 cells and MDA-MB-231cells were cultured in 24-well plates (0.5×10^5/well). Each transfected with 30 ng of pMIR-BECN1 3'UTR or 200 ng of PGL3-DRAM1 3′UTR, together with 5 ng pRL-SV40 vector (Promega), which contains the Renilla luciferase gene, used to normalize transfection efficiency, and 100 nM of miR-199a-5p mimic or Negative control (NC) (GenePharma). Transfection was done using Lipofectamine™ 2000 (Invitrogen). At 36 h or 48 h after transfection, firefly and Renilla luciferase activities were examined using the Dual-Luciferase Reporter Assay (Promega). Each transfection was repeated in Quintuplicate. The experiment was done thrice independently.

2.8. Western blot analysis

MCF7 Cells were harvested at 20 h after irradiation and MDA-MB-231 cells were harvested at 16 h after irradiation (based on the time-course curve). Cell pellets were lysed in RIPA lysis buffer. 30 or 60 μg of total protein was separated by SDS–PAGE, transferred to nitrocellulose membrane, and analyzed by immunoblotting using the chemiluminescence (Santa Cruz). The primary antibodies used were DRAM1 (1:300) (Abcam) and LC3-I/II (1:300) (Cell Signaling), GAPDH (1:1000) and BECN1 (1:1000) (Santa Cruz). The autophagic flux was analysised by Western blot to detect MAPLC3 expression of breast cancer cells treated with 20 μM chloroquine (CQ) (Sigma). The intensity of protein bands were quantified using image j software and the ratio of specific band to control was analyzed.

2.9. GFP-LC3 localization assay

In order to generate stable expression of GFP-LC3 in MCF7 cells, we transiently co-transfected pQN-GFP-LC3 vector (10 μg) and Amphopack (10 μg) plasmid into packaging cell line of 293T. The pseudoviral particles were purified 72 h post-transfection and mixed with polybrene (8 μg/ml), the mixtures were used to infect MCF7 cells. 1000 μg/ml G418 was used for selection until positive colonies acquired. MCF7 cells stably expressing GFP-LC3 were planted at a density of (1×10^5) in 6-well plate with glass coverslips and exposed to the indicated transfections of microRNA and IR. Cells were then stained by methanol for 10 min. GFP-LC3 puncta were visualized under an inverted fluorescence (Olympus XSZ-D2) microscope equipped with CCD cameras and stack of images were captured and analyzed for presence of more than five puncta per cell.

2.10. Cell viability assay

Cells were seeded at a density of 4×10^3 in 96-well plates. 24 h after the transtection of miRNAs (100 nM) cells were treated with IR. 72 h later, 100 μl Cell Counting Kit-8 (CCK8) (Dojindo, Beijing, China) solution were added to each well and the plates were incubated at 37 °C for 4 h. Absorbance at 560 nm was measured using a microplate spectrophotometer (BioTek™). Absorbance of cell survival was calculated relative to control cells, which were set to 100%. Each transfection was repeated in Quintuplicate.

2.11. FACS assay

For cell cycle detection, cells were plated into 6-well plates (2×10^5/well) and treated with miRNAs 48 h or combined with IR treatment. Cells were washed with PBS and stained in the dark with 50 μg/ml phosphatidyl inositol (PI) and 0.1% ribonuclease A (RNase A) in 400 μl of PBS for 15 min, cells were then analyzed by using FACSort Flow Cytometer (Becton Dickinson).

2.12. Statistical analysis

Statistical evaluations are presented as mean ± S.E. Data were analyzed using the Student's *t*-test or χ^2 test for statistical significance. *P* values were considered significant if $P < 0.05$.

3. Results

3.1. Overexpression of miR-199a-5p inhibits radiation-induced autophagy in MCF7 cell line

Synthetic miR-199a-5p (mimic) was added to MCF7 cells and quantitative real time-PCR (qRT-PCR) was performed to ensure successful overexpression of miRNA. As shown in (Fig. 1A), miR-199a-5p level was enhanced to more than 20 folds after transfection of MCF7 cells with mimic relative to NC transfected cells. During autophagy process, the mammalian ATG8-homologue (LC3-I) is processed and recruited to the autophagosomes, where the lipdated (LC3-II) is generated [26]. To examine the effect of miR-199a-5p on autophagy, we stably transfected MCF7 cells with GFP-LC3 plasmid to monitor autophagosome formation through direct fluorescence microscopy, measured as an increase in puncta positive cells (Fig. 1B). To trigger autophagy, we used ionizing radiation (IR) which has been shown to induce autophagy effectively in diverse tumor cells including breast cancer cells [27]. Consistently, IR significantly increased the number of puncta-positive cells in mock (non-transfected) and NC transfected MCF7 cells ($^{**}P < 0.01$). Importantly, upon ectopic overexpression of miR-199a-5p, only a limited number of irradiated MCF7 cells were able to form autophagosomes (Fig. 1C, $^{**}P < 0.01$). Next, we examined the expression of LC3-II protein by Western blot analysis and found that IR enhanced LC3-II protein level which was suppressed upon ectopic overexpression of miR-199a-5p (Fig. 1D). Both inhibition of autophagosome formation and excessive autophagosomes degradation can result in reduction of LC3-II [28,26]. To distinguish between these two possibilities, we used chloroquine (CQ) [26], an agent that impairs lysosomal acidification, to inhibit LC3-II degradation and thereby detect the autophagic flux [28]. As shown in (Fig. 1E), miR-199a-5p inhibited IR-induced autophagy as represented by decreased LC3-II/I conversion ratio. After IR exposure, LC3-II accumulation was markedly increased in CQ-treated NC transfected cells (Fig. 1E), whereas it was only minimally altered in miR-199a-5p transfected cells, indicating the diminished conversion of LC3-I to LC3-II. These data support that the decrease of LC3-II by miR-199a-5p resulted from the inhibition of autophagosome formation and not from excessive autophagosome degradation. Therefore, miR-199a-5p is a bona fide inhibitor of IR-induced autophagy in MCF7 breast cancer cells.

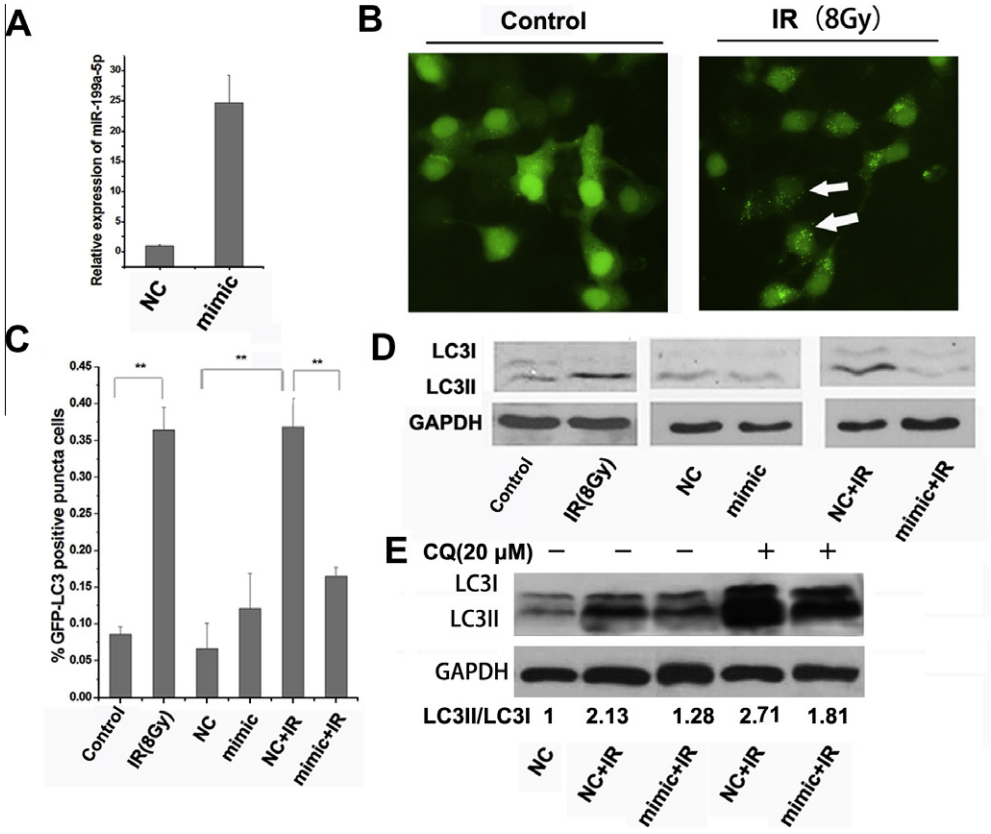

Fig. 1. Overexpression MiR-199a-5p suppresses IR-induced autophagy in MCF7 cells. (A) miR-199a-5p mimic and NC were transfected into MCF7 cells and qRT-PCR was used to detect the miR-199a-5p level. (B) Representative images of MCF7 cells stably expressing GFP-LC3 after the treatment of irradiation, left: sham-irradiation, GFP-LC3 distributes evenly (autophagy was not induced), right: white arrow pointing at typical puncta positive cell (induction of autophagy by irradiation). (C) MCF7 cells with stable expression of GFP-LC3 were transfected with miR-199a-5p mimic (100 nM) or NC (100 nM), percentage of total GFP puncta positive cells were quantitated (undergoing IR-induced autophagy). The error bars indicate the standard error of the mean (S.E.M.) for three independent experiments (** $P < 0.01$). (D) Western blot analysis of MAPLC3 expression in MCF7 cells after transfection with or without irradiation. (E) After transfection, MCF7 cells were treated with CQ (20 μM) for 1 h before exposure to irradiation, then MAPLC3 was detected by Western blot.

3.2. Overexpression of miR-199a-5p suppresses DRAM1 and Beclin1 expression in MCF7 cell line

To explore the underlying mechanism by which miR-199a-5p inhibited autophagy, we combined the database from three popular microRNA target prediction programs (miRBase, PicTar and Targetscan), searching for the putative autophagy-related target genes. As a result, we found that *DRAM1* and *Beclin1* genes were good candidates, as they contain the matched nucleotides to the seed sequence of miR-199a-5p (Fig. 2A and B). *DRAM1* has been demonstrated to promote autophagy [29], while *Beclin1* is well appreciated determinant gene in initiation of autophagy [30]. To provide experimental evidence supporting that *DRAM1* or *Beclin1* is a target of miR-199a-5p, we cloned the partial 3′UTR of *DRAM1* or *Beclin1* containing miR-199a-5p-binding sequence to firefly luciferase reporter vector. We examined the effects of miR-199a-5p on the luciferase activity at these regions by using miR-199a-5p mimic. Luciferase reporter assay indicated that miR-199a-5p significantly inhibited ($^*P < 0.05$) the luciferase activity in the reporter vector containing wild-type 3′UTR of *DRAM1* or *Beclin1*, but not in the mutant 3′UTR vectors, demonstrating the specificity of miR-199a-5p on *DRAM1* and *Beclin1* 3′UTR targeting (Fig. 2C and D). We also examined the impact of miR-199a-5p on endogenous *DRAM1* or *Beclin1* protein levels in MCF7 cells. The results showed that an ectopic increase in miR-199a-5p expression in MCF7 cells led to marked reduction in *DRAM1* and *Beclin1* protein content. Importantly, exposure of MCF7 cells to IR led to up-regulation of *DRAM1* and *Beclin1* protein expression levels. MiR-199a-5p overexpression obviously attenuated such IR stimulatory effect on *DRAM1* and *Beclin1* expression levels (Figs. S1A and S1B; Fig. 2E and F). These data suggested that miR-199a-5p suppressed IR-induced autophagy by directly targeting *DRAM1* and *Beclin1* in MCF7 cells.

3.3. Overexpression of miR-199a-5p up-regulates autophagy, DRAM1, and Beclin1 in MDA-MB-231 breast cancer cell line

We next sought to explore the impact of miR-199a-5p overexpression on autophagy in another breast cancer cell line, MDA-MB-231 cells. On contrary to MCF7 and rather surprisingly, we found that upon overexpression of miR-199a-5p in MDA-MB-231 cells, LC3-II/LC3-I conversion ratio was increased as compared to NC. Furthermore, miR-199a-5p promoted the IR-induced autophagy in this cell line (Fig. 3A, Fig. S1D). To confirm the results, we measured the autophagic flux. Pre-treatment of MDA-MB-231 cells with CQ enhanced LC3-II expression, which was further increased upon miR-199a-5p overexpression (Fig. 3B). These results indicate that miR-199a-5p promotes autophagosome formation. Therefore miR-199a-5p behaves as an autophagy inducer in MDA-MB-231 cell line. Such positive relation between miR-199a-5p and autophagy in MDA-MB-231 cells triggered us to examine the impact of miR-199a-5p on the expression of its target genes *DRAM1* and *Beclin1* in MDA-MB-231. Surprisingly, we found that ectopic overexpression of miR-199a-5p in MDA-MB-231 cells led to drastic increase in expression level of *DRAM1* and *Beclin1* proteins as indicated by Western blotting (Fig. 3C). Although exposure of MDA-MB-231 cells to IR led to increased *DRAM1* and *Beclin1* protein expression levels, miR-199a-5p overexpression did not further enhance the *DRAM1* and *Beclin1* expression levels in irradiated

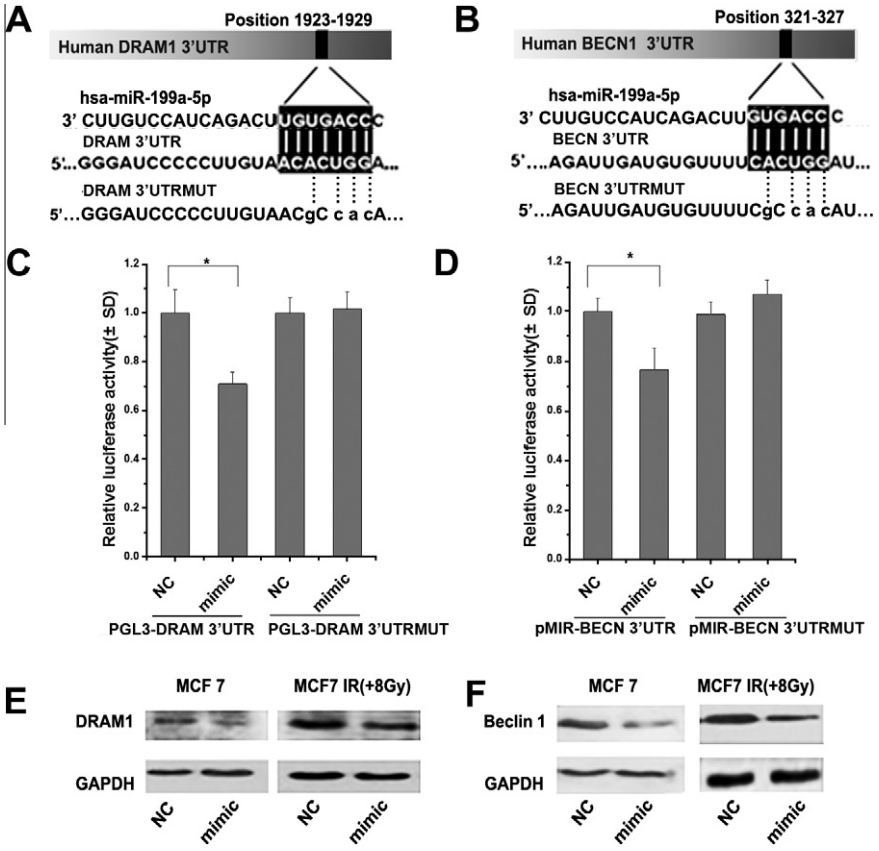

Fig. 2. MiR-199a-5p down-regulates DRAM1 and Beclin1 expression in MCF7 cells. (A) and (B) Sequence alignment between miR-199a-5p and the 3′UTR of *DRAM1* and *Beclin1*. Solid line shows the seed match region. (C) and (D) Luciferase activity analysis of *DRAM1* and *Beclin1* 3′UTR (wild type and mutant constructs) were performed after co-transfection with miRNA in MCF7 cells. The error bars indicate the standard error of the mean (S.E.M.) for three independent experiments ($^*P < 0.05$). (E) and (F) Western blot analysis DRAM1 and Beclin1 expression in MCF7 cells after transfection or IR.

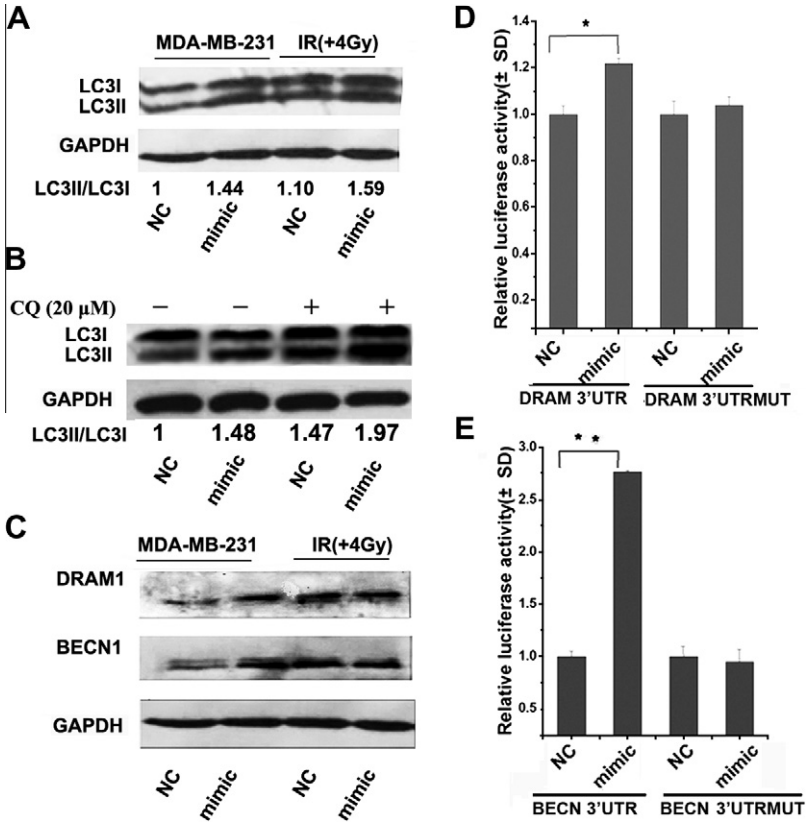

Fig. 3. MiR-199a-5p overexpression enhanced autophagy and up-regulated DRAM1 and Beclin1 expression in MDA-MB-231 cells. (A) Western blot analysis of MAPLC3 expression in MDA-MB-231 cells after transfection with miRNA or treated with IR. (B) After transfection, MDA-MB-231 cells were treated with CQ (20 μM) and MAPLC3 was detected by Western blot. (C) Western blot was used to detect the expression of DRAM1 and Beclin1 in MDA-MB-231 cells following the indicated treatments. (D) and (E) Luciferase activity analysis of *DRAM1* and *Beclin1* 3′UTR (wild type and mutant constructs) was performed after co-transfection with miRNA in MDA-MB-231 cells. The error bars indicate the standard error of the mean (S.E.M.) for three independent experiments ($^{**}P < 0.01$, $^{*}P < 0.05$).

MDA-MB-231 cells (Fig. S1C; Fig. 3C). To explore the possible underlying mechanism, we co-transfected plasmids carrying *DRAM1* or *Beclin1* 3′UTRs containing the binding site for miR-199a-5p. Luciferase activity with *DRAM1* 3′UTR and *Beclin1* 3′UTR constructs increased significantly in the miR-199a-5p mimic MDA-MB-231 transfected cells ($^{*}P < 0.05$, $^{**}P < 0.01$), and mutation in the miR-199a-5p target genes sequence led to complete abrogation of the stimulatory effect (Fig. 3D and E). These data suggest that miR-199a-5p up-regulates *DRAM1* and *Beclin1* expression directly through targeting 3′UTR of *DRAM1* and *Beclin1* mRNA to promote basal and IR-induced autophagy in MDA-MB-231 cells.

3.4. MiR-199a-5p regulates IR-induced cell cycle checkpoint

Since it has been suggested by Vasudevan et al. [31] that miRNAs could repress their target genes in proliferating cells and activate their target genes in arrested cells, we sought to explore whether miR-199a-5p could affect the cell cycle dynamics in both cell lines. As shown in (Fig. 4A), overexpression of miR-199a-5p induced accumulation of cells at G2/M phase in MDA-MB-231 cell line, but not in MCF7 cells. Exposure to IR led to decreased proportion of cells at G0/G1 peak and increased proportion of cells at G2/M peak in both cell lines. Strikingly, we found that IR-induced cell cycle arrest in MDA-MB-231 and MCF7 cells was abolished by miR-199a-5p overexpression as analyzed by the flow cytometry assay. These results indicate that miR-199a-5p overexpression induces changes in cell proportions pre-IR in MDA-MB-231 cell line and impairs IR-induced cell cycle arrest in MDA-MB-231 and MCF7 cell lines.

3.5. MiR-199a-5p altered the radiation responsiveness of breast cancer cells

Since we found that miR-199a-5p could abolish the IR-induced cell cycle changes, we hypothesized that modulation of miR-199a-5p could alter the radiosensitivity of the breast cancer cell lines. First we investigated whether IR could have an impact on miR-199a-5p expression profile. Using quantitative qRT-PCR, we found that endogenous miR-199a-5p expression was enhanced by IR in MCF7 cells but was decreased in MDA-MB-231. After transfection with mimic, miR-199a-5p expression was up-regulated and further enhanced by IR in both cell lines (Fig. 4B and C). To determine if miR-199a-5p mimic could modulate the radiation sensitivity of breast cancer cells, we performed cell viability assay. In MDA-MB-231 cell line, we found that miR-199a-5p mimic radiated group had significantly decreased cell viability compared to NC radiated group (Fig4D). In MCF7 cell line, miR-199a-5p overexpression did not affect the radiosensitivity significantly (data not shown). These results are consistent with the hypothesis that miR-199a-5p overexpression induces radiation sensitivity of breast cancer cells.

4. Discussion

The rapid advancement in our understanding of the mechanisms and regulation of autophagy has placed this process at the center of current research in major human disorders especially cancer. Despite that, a huge gap in molecular control of autophagy still exists [1,32,33]. The novel endogenous gene regulators,

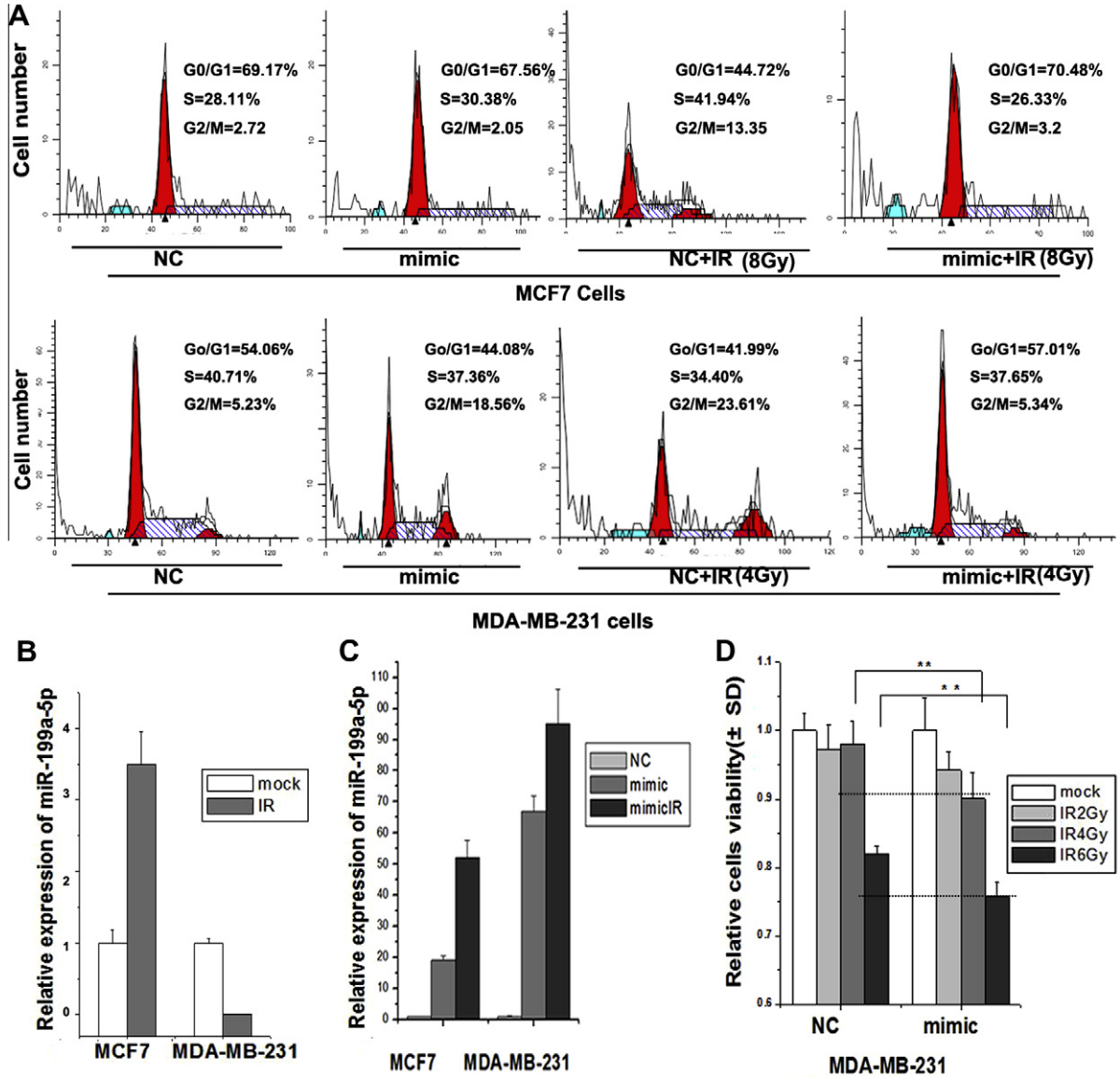

Fig. 4. miR-199a-5p overexpression enhanced the radiosensitivity of MDA-MB-231 cells. (A) Flow cytometry analysis depicted cell-cycle progression of MCF7 and MDA-MB-231 cells after overexpression of miR-199a-5p or exposure to IR. (B) and (C) qRT-PCR was used to measure the relative expression level of miR-199a-5p after transfection with mimic and NC or exposure to IR. (D) CCK8 assay was implemented to measure the cell viability in MDA-MB-231 cells after the indicated treatment. The error bars indicate the standard error of the mean (S.E.M.) for three independent experiments ($^{**}P < 0.01$).

miRNAs, have been implicated in fundamental cellular activities including growth, development, apoptosis and cancer. Modulation of autophagy through miRNAs is a novel area of research and still in its infancy. Several miRNAs have been demonstrated to control autophagy process via targeting the autophagy related genes in diverse human cancer cells [16,17,34,35], These studies helped to understand autophagy signaling in depth and also offered novel therapeutic perspectives. Ectopic overexpression of miR-30a in chronic myelogenous leukemia (CML) cells abrogated the Imatinib-induced autophagy via suppression of two target genes Beclin1 and ATG5 to ultimately enhance the cytotoxic effect of imatinib-induced apoptosis [36]. Interestingly, autophagy has been reported to control miRNA biogenesis and activity, suggesting a feedback loop between miRNAs and autophagy [37]. In our study, we found that miR-199a-5p overexpression led to suppression of IR-induced autophagy in MCF7 breast cancer cell line. However, probably due to low basal autophagic activity in MCF7, we could not notice an obvious decrease in percentage of puncta positive cells nor in LC3-II by overexpressing miR-199a-5p pre-IR. Using in silico analysis, we found *DRAM1* and *Beclin1* were possible targets of miR-199a-5p. Recently, studies have shown that *DRAM1* belongs to an evolutionarily conserved family of proteins, and encodes a series of p53-inducible splice variants, part of which localize to peroxisomes and autophagosomes and are required for p53-induced autophagy [29,38–41]. The other putative target gene, *Beclin1*/*ATG6* is the widely studied autophagy related gene. *Beclin1* has a central role in autophagy machinery and play as a key autophagy-promoting gene, including IR-induced autophagy [16,27,42,43]. Using luciferase assay and Western blotting, we showed that both *DRAM1* and *Beclin1* are novel target genes for miR-199a-5p. Overexpression of miR-199a-5p in MCF7 cells suppressed the expression of *DRAM1* and *Beclin1* via targeting the 3′UTR of these genes. Added to this, miR-199a-5p could successfully suppress the expression of *DRAM1* and *Beclin1* proteins in MCF7 cells in the presence or absence of IR. Collectively, these finding imply that miR-199a-5p potently suppresses IR-induced

autophagy in MCF7 cells through its inhibitory effect on *DRAM1* and *Beclin1* at least partially, since a single miRNA could target several genes simultaneously. Very recently, it has been reported that miR-199a-5p targets and inhibits autophagy-associated gene 7 (ATG7) to suppress Cisplatin-induced autophagy in liver cancer cells [44]. In MDA-MB-231, which is characterized by being highly invasive estrogen-receptor negative breast cancer cell line, while MCF7 are non-invasive estrogen-receptor negative cells [45], we showed that miR-199a-5p behaved in a totally opposite trend. Overexpression of miR-199a-5p enhanced both basal and IR-induced autophagy in this cell line. Similarly, miR-199a-5p ectopic overexpression resulted in sharp up-regulation of *DRAM1* and *Beclin1* target genes expression through directly targeting 3′UTR of *DRAM1* or *Beclin1* mRNA in MDA-MB-231 cells. From the enormous body of literature in the field of miRNAs, we found only countable number of studies reported that miRNAs could, through different mechanisms, up-regulate rather than suppress gene expression. In human liver cells, miR-122 was found to bind to 5′UTR of hepatitis C virus RNA and activate its translation [46]. MiR-10a was found to bind to 5′UTR segment of ribosomal protein mRNA, leading to stimulation of ribosomal protein mRNA translation and ribosome biogenesis and ultimately up-regulate global protein synthesis [47]. We excluded this possible mechanism by blasting miR-199a-5p and the 5′UTR sequence of *DRAM1* and *Beclin1*, and we found there were no potential binding sites. The other mechanism involved interaction between the promoter region of the gene and specific miRNA [48]. We also excluded this possible mechanism by blasting miR-199a-5p and the promoter sequence of *DRAM1* and *Beclin1*, and we found there were no potential binding sites. Steitz and Vasudevan performed a series of studies to demonstrate the ability of miRNAs to activate gene translation by targeting the 3′UTR. The authors demonstrated that cell cycle cues determine whether miRNAs activate or repress target genes. They suggested that miRNAs could activate gene translation in quiescent phase (cells arrested at G0/G1 phase of cell cycle), which was brought about by serum starvation or contact inhibition, and repress translation in the later stages of the cell cycle [49–52]. Such phenomenon has been found to occur naturally in *Xenopus laevis* oocytes [53]. From this perspective, we sought to explore whether miR-199a-5p induces G0/G1 arrest in order to up-regulate its target genes. However, we found that miR-199a-5p induced accumulation of cells at G2/M peak in MDA-MB-231 but not in MCF7 cell line. After exposing both cell lines to IR, proportion of cells increased at G2/M and decreased at G0/G1, such event was completely reversed upon overexpression of miR-199a-5p in both cell lines. The forth possibility argues that miRNA-mediated gene activation could be cell-line specific feature. In MIA PaCa-2 pancreatic cancer cells, MiR-21 ectopic overexpression led to significant up-regulation of Bcl-2 target gene expression by targeting the 3′UTR of Bcl-2 mRNA, while it was documented that miR-21 suppresses Bcl-2 expression in breast cancer cells also via targeting Bcl-2 3′UTR [54,55]. Similarly, via direct action on 3′UTR of Krüppel-like factor 4 (KLF4) mRNA, overexpression of miR-206 promoted KLF4 gene expression in MCF10A mammary epithelial cells, while it suppressed expression of KLF4 in MDA-MB-231 breast cancer cells [56]. Collectively, it seems that the impact of miR-199a-5p on *DRAM1* and *Beclin1* genes could be also cell line specific. Of course, further comprehensive investigations are warranted. Overall our findings add more interest and challenge to further understand the mechanisms of miRNAs, especially regarding how miRNAs regulate the gene expression which is still largely illusive [57]. Next we showed that IR up-regulated miR-199a-5p expression in MCF7 and down-regulated miR-199a-5p expression in MDA-MB-231 cells. After transfection with mimic, miR-199a-5p expression was enhanced pre-IR and further enhanced post-IR in MCF7 cells. However, we did not observe a decrease of miR-199a-5p in MDA-MD-231 cell line in response to IR probably due to very high levels of miR-199a-5p after transfection with mimic, similar to [58]. The underlying mechanism of miR-199a-5p radio-response could involve ATM activation which phosphorylates KSRP (KH-type splicing regulatory protein), the key component in both Drosha and Dicer miRNA-processing complexes, to ultimately enhance miR-199a and other miRNAs biogenesis [59]. Finally, using cell viability assay, we showed that miR-199a-5p overexpression enhanced the radiosensitivity of MDA-MB-231 cell line. Several studies have demonstrated the significance of miRNA modulation to improve the radio- or chemotherapy, holding promising hope to improve the anti-tumor efficacy [60–63]. However, there is a big gap in understanding the detailed mechanisms and intracellular pathways through which miRNAs exert their effects. Thus, further extensive basic research will be needed to fully lay open the whole number of miRNAs involved in modulation of chemo- or radiotherapies and the way they affect cellular homeostasis. Moreover, it is important to validate the safety and efficiency of such treatment combinations in clinical settings [64].

Briefly, we reported for the first time that miR-199a-5p is a novel regulator of basal and IR-induced autophagy in human breast cancer cells. Moreover, we found that both *DRAM1* and *Beclin1* are novel target genes, through which miR-199a-5p could probably regulate autophagy. Uniquely, we demonstrated dual differential roles of miR-199a-5p in autophagy and target gene expression in two different human breast cancer cell lines. Collectively, our findings provide evidence for a new role of miR-199a-5p in a cellular process that play significant part in carcinogenesis and cancer therapy, which will ultimately assist in better understanding of miRNA-modulated autophagic signaling networks and thereby improve the current and future cancer therapeutic strategies.

Acknowledgments

We are grateful to the staff at the Key Laboratory of Radiobiology (Ministry of Health), this study was supported by NSFC Grant (30770649, 30970682), Research Fund for the Doctoral Program of Higher Education of China (20100061110070), Program for New Century Excellent Talents in University, the Fundamental Research Funds for the JiLin University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.12.027.

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