MiR-200c inhibits autophagy and enhances radiosensitivity in breast cancer cells by targeting UBQLN1

Quanquan Sun1*, Tongxin Liu1*, Yawei Yuan1, Zhenli Guo2, Guozhu Xie3, Shasha Du1, Xiaoshan Lin1, Zhixin Xu1, Minfeng Liu3, Wei Wang1, Quan Yuan3 and Longhua Chen1

1 Department of Radiation Oncology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong Province, People’s Republic of China
2 Department of Pathology, Anhui Provincial Hospital of Anhui Medical University, Hefei, Anhui Province, People’s Republic of China
3 Department of Breast Center, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong Province, People’s Republic of China
4 Jules Stein Eye Institute, David Geffen School of Medicine, University of California, Los Angeles, CA

Breast cancer is the most common cancer in women worldwide.1 Radiotherapy is an important part of the treatment in most patients receiving breast-conserving surgery and displays significant clinical benefits, such as decreasing the risk of local recurrence and reducing the risk of mortality due to breast cancer.2 However, for certain subtypes of breast cancer (e.g., basal-like), the local and regional control remains unsatisfactory. A major reason for this failure in treatment may be due to its radioresistance.3–5 Therefore, understanding the molecular mechanisms involved in the radioresistance of breast tumors may lead to improved clinical outcomes.

Autophagy is a cellular process that involves self-degradation and recycling of macromolecules and cellular organelles.6,7 It is, in most circumstances, a prosurvival mechanism under stressful conditions. Autophagy has been implicated in a variety of human diseases.7–9 Similar to the situation in normal cells, autophagy is also critical for tumor cells to survive stressful conditions, and thus has been implicated in tumor resistance to chemotherapy and radiotherapy.10–13

MicroRNAs (miRNAs) regulate a variety of biological processes, including cell proliferation, differentiation and invasion.14 Dysregulation of miRNAs has been reported to contribute to cancer,15,16 and implicated in chemoresistance and radioresistance via modulation of autophagy.10,13 Such findings are not surprising considering the fact that miRNAs are key regulators of autophagy.17

The miR-200 family is involved in the self-renewal of cancer stem cells,18 epithelial-to-mesenchymal transition (EMT)19,20 and chemosensitivity.21 Recent studies indicated that miR-200c, the prevailing member of the miR-200 family,19,20,22,23 could sensitize cancer cells to radiation by targeting TBK1 and VEGF-VEGFR2, despite the unspecified relationship between miR-200c and autophagy.24,25

The results from our study showed that miR-200c could sensitize breast cancer cells to radiation via a mechanism associated with inhibition of irradiation-induced autophagy.
We also identified ubiquilin 1 (UBQLN1) as a link between miR-200c, autophagy and radioresistance of breast cancer cells. These results provide new insights into the molecular functions of miR-200c in radiosensitivity in breast cancer cells and imply a rationale for enhancing radiosensitivity via miR-200c for the treatment of breast cancer.

Material and Methods

Patient tissue and cell culture
Breast cancer specimens were collected from 35 patients who receive surgical resection between October 2011 and December 2012. The study protocol was approved by the Institutional Review Board at Nanfang Hospital, Southern Medical University (Guangzhou, China).

MCF-10A cells were from American Type Culture Collection (ATCC). All other cell lines were from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MDA-MB-231, BT549 and BT474 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco, Australia), 100 U/ml penicillin and 100 mg/ml streptomycin. MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. MCF-10A cells were cultured in DMEM/F12 (1:1) medium supplemented with 5% horse serum (HyClone, Logan, UT), 100 ng/ml cholera toxin (Sigma-Aldrich, St. Louis, MO), 20 μg/ml epidermal growth factor (EGF; Peprotech, Rocky Hill, NJ), 0.5 μg/ml hydrocortisone (Sigma-Aldrich) and 10 μg/ml insulin (Sigma-Aldrich). All cells were cultured in a humidified air containing 5% CO2 at 37°C.

Quantitative real-time polymerase chain reaction
Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The reverse-transcription and polymerase chain reaction (PCR) primers for miR-200c and U6 were from RiBoBio (Guangzhou, China). The PCR primers for UBQLN1 were 5’-AGAGGCGAGGTTACCCACCA-3’ and 5’-TTA TGCCCTCCAGGTAACTTTTG-3’. The PCR primers for β-actin were 5’-CTGACACGGCTCCGAGCAT-3’ and 5’-AAGGTTGCTGCAGATTTC-3’. The cDNA library was synthesized using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). For quantification of mature miRNA, cDNA was generated using specific stem-loop universal primers. Real-time (RT)-PCR for miRNA and mRNA was performed using SYBR Premix Ex Taq II (TaKaRa) and was measured using an ABI 7500 Sequence Detection System (Perkin Elmer/Applied Biosystems, Rotkreuz, Switzerland). U6 or β-actin was used as the internal control.

Oligonucleotides and siRNA transfection
The miRNA mimic, inhibitors, the scrambled negative control oligonucleotides, UBQLN1 and ATG7 siRNA were from RiBoBio. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacture’s protocol. For efficient inhibition of the miR-200bc/429 cluster, equivalent amounts of miR-200c and miR-429 inhibitors were combined.

Irradiation and clonogenic assay
At 48 hr after transfection with oligonucleotides or siRNA, increasing numbers of cells were seeded on six-well plates in triplicate and exposed to radiation at indicated doses using 6 MV X-ray generated by a linear accelerator (Varian 2300EX, Varian, Palo Alto, CA) at a dose rate of 5 Gy/min. After incubation at 37°C for 10–14 days, the cells were fixed using 100% methanol and stained with 1% crystal violet. Colonies containing >50 cells were counted under a light microscope. The surviving fraction was calculated as described previously.26 In some experiments, cells were treated with 3-methyladenine (3-MA; Sigma-Aldrich) or bafilomycin A1 (Baf. A1; Abcam, San Francisco, CA, USA), starting from 1 hr before radiation, for a duration of 24 hr.

Antibodies and Western blot analysis
The primary antibodies used in our study included: rabbit anti-LC3B, anti-p62/SQSTM1, anti-cleaved PARP (Cell Signaling Technology, Beverly, MA, USA), anti-active caspase-3 (Abcam), anti-UBQLN1 (Abnova, Taipei, Taiwan) and mouse anti-β-actin (ProteinTech, Chicago, IL, USA). Western blot analysis was performed as described previously.26 Briefly, whole-cell lysate was prepared using radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology) containing proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentration was determined using a bicinchoninic acid protein assay (Pierce Bio-technology, Rockford, IL). Proteins were resolved using sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad, Rockford, IL).
Cell proliferation assay
Cell proliferation assay was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method, as described previously. Briefly, cells (2 × 10^5 cells per well) were plated into 96-well culture plates. After 24-hr incubation, 3-MA (final concentration 5 mM) was added to each well and incubated for an additional 24 hr before 2-h incubation with 50 μl of MTT solution (2 mg/ml; Sigma-Aldrich) at 37°C. The medium was aspirated, and the dark blue crystals were dissolved by adding 100 μl dimethyl sulfoxide. The absorbance was measured at 570 nm. For treatment with ATG7 siRNA, UBQLN1 siRNA or miR-200c mimic and negative control with scrambled sequence, cells were seeded 48 hr after transfection and incubated for 1–3 days. Each experiment was performed for at least three times, each with triplicate samples.

Preparation of stable GFP-LC3-expressing cells
A recombinant lentivirus containing GFP-LC3 was obtained from GenePharma (Shanghai, China). MDA-MB-231 cells were infected with lentivirus particles and isolated via fluorescence-activated cell sorting to obtain the cells stably expressing GFP-LC3.

Flow cytometric analysis of apoptosis
Cells were seeded on six-well plates and transfected with the miRNA mimic scrambled negative control or the miR-200c mimic 48 hr before irradiation, for a duration of 24 hr. At least three independent experiments were performed using the Dual-Luciferase Assay kit (Promega) and normalized to firefly luciferase activity. Three independent experiments were performed in triplicate.

UBQLN1 rescue experiment
A pEZ-M02-UBQLN1 plasmid in which the UBQLN1 cDNA does not contain the 3’UTR targeted by miR-200c and an empty vector plasmid were synthesized by GenePharma (Shanghai, China). Cells were co-transfected with miR-200c mimics or negative control miRNA and with pEZ-M02-UBQLN1 plasmid or the empty vector plasmid. Clonogenic assay was carried out at 48 hr after the transfection. The UBQLN1 expression and irradiation-induced autophagy activity were verified with Western blot analysis.

Immunohistochemical staining
Immunohistochemical (IHC) staining was performed on 3-μm-thick sections prepared using tissue blocks embedded in paraffin. The sections were fixed using 4% paraformaldehyde overnight, embedded in paraffin, followed by deparaffinization and hydration. The sections were pretreated with a sodium citrate buffer in a microwave for antigen retrieval and blocked using normal goat serum. The sections were stained using rabbit anti-UBQLN1 (Abnova, 1:100 dilution) or anti-LC3A/B (Cell Signaling Technology, 1:100 dilution) overnight at 4°C, and then incubated in a biotinylated goat anti-rabbit IgG secondary antibody for 1 hr, followed by staining with an avidin-biotin peroxidase complex (GeneTex, Irvine, CA, USA). Scoring of the sections was performed by two independent pathologists.

In situ hybridization
For in situ hybridization (ISH), the sections were deparaffinized using xylene, rehydrated in serial dilutions of ethanol and treated with 0.2 N HCl for 5 min. After washing for three times, the sections were incubated in proteinase K (40 μg/ml, Promega) for 20 min. After extensive washing in PBS containing 0.2% glycine, the sections were fixed using 4% paraformaldehyde for 10 min. The sections were reconstituted using hybridization solution and incubated at 56°C overnight in a digoxigenin-labeled LNA-miR-200c probe (Exiqon, Vedbaek, Denmark). The sections were washed twice with 5× SSC, each for 20 min at room temperature, followed by three washing with 2× SSC for 20 min at 50°C. Sections were then blocked with 5% normal goat serum for 1 hr at room temperature before incubation in an anti-digoxigenin alkaline

Hercules, CA, USA) electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 5% nonfat milk for 1 hr before incubation with a primary antibody overnight at 4°C. After extensive washing, the membranes were incubated in an appropriate horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. The protein band of interest was visualized using an ECL method (Pierce Biotechnology).
phosphatase conjugate (Roche, Stockholm, Sweden) overnight at 4°C. After staining with 5-bromo-4chloro-3indolyl phosphate (BCIP)/nitroblue tetrazolium chloride (NBT) buffer for 10 min, colorimetric signals were obtained by incubating the sections in BCIP/NBT buffer in the dark for 4 hr at room temperature. Nuclear fast red was used as the counterstain. For the negative control, digoxigenin-labeled LNA-scrambled miRNA was used. Scoring of the sections was performed by two independent pathologists as negative (−), focally or weakly positive (+) or strongly positive (++).

Statistical analysis
Data obtained from experiments using cultured cells are presented as the means ± standard deviation, and analyzed using Student’s t-test or analysis of variance (ANOVA). A Fisher’s test or Student’s t-test was used to analyze the relationship between miR-200c, UBQLN1 and LC3 expression and the clinical characteristics. All statistical analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL), and a p value of <0.05 was considered to be statistically significant. Qualitative data were representative of more than three independent experiments, with each performed in triplicate.

Results
Mir-200c sensitizes breast cancer cells to radiation treatment
In our study, we first investigated miR-200c expression in four breast cancer cell lines and the normal breast epithelial cell line MCF-10A. Consistent with a previous report, expression of miR-200c was higher in luminal phenotype breast cancer cells (MCF-7 and BT474) and lower in basal phenotype breast cancer cells (MDA-MB-231 and BT549) compared to the normal breast epithelial cell line MCF-10A (p < 0.05, Fig. 1a).

Next, we investigated the effect of miR-200c on the radiosensitivity of breast cancer cells. Overexpression of miR-200c reduced the survival fraction of the MDA-MB-231 and BT549 cell lines subjected to irradiation (0–8 Gy). In contrast, inhibiting the expression of miR-200c increased the survival fraction in MCF-7 cells postirradiation (Figs. 1b and 1c, Supporting Information Table 1).

Autophagy is induced by irradiation and acts as a protective mechanism in breast cancer cells
Autophagy may contribute to either cytoprotective or cytotoxic effects, depending on the type of cell and stress.11,29–31 In our study, irradiation activated autophagy in MDA-MB-231, BT549 and MCF-7 cell lines, as reflected by increased expression of LC3II and degradation of p62 (Fig. 2a). However, irradiation-induced autophagy activity in MCF-7 cells was not significantly clear in comparison to the MDA-MB-231 and BT549 cell lines. 3-MA, an autophagy inhibitor that blocks autophagosome formation, significantly reduced cell viability and enhanced the radiosensitivity of three human breast cancer cell lines (MDA-MB-231, BT549 and MCF-7) (Figs. 2b and 2c, Supporting Information Fig. S1 and
Supporting Information Table 2). Knockdown of ATG7, which is a core autophagy-associated gene, also reduced cell viability, suppressed irradiation-induced autophagy and enhanced radiosensitivity of breast cancer cells (Supporting Information Fig. S2 and Supporting Information Table 3). These results indicate that autophagy is activated due to an adaptive response to promote the survival of breast cancer cells after receiving irradiation.

**MiR-200c inhibits irradiation-induced autophagy**

In MDA-MB-231 cells stably expressing the GFP-LC3 fusion protein, miR-200c overexpression significantly decreased the lipidation of LC3 and degradation of p62 protein induced by irradiation (Figs. 3a and 3b). To further investigate the specific stage of autophagy that was inhibited by miR-200c, we used bafilomycin A1, a fusion inhibitor of lysosomes and autophagosomes, which can inhibit the degradation of LC3II. Twenty-four hours after irradiation, the bafilomycin A1-treated scrambled negative control cells displayed markedly increased accumulation of LC3II, whereas the ectopic expression of miR-200c attenuated such a response (Fig. 3c). These data indicate that the inhibitory effect of miR-200c on autophagy resulted from the suppression of the early stage of autophagy, the inhibition of autophagosome formation, rather than from the suppression of autophagosome degradation.

Next, we sought to determine whether miR-200c-induced radiosensitization was dependent on suppression of autophagy. In nonirradiation groups, neither 3-MA nor miR-200c mimic alone affected the apoptosis rate in MDA-MB-231 cells ($p > 0.05$, Supporting Information Fig. S3). 3-MA in combination with miR-200c mimic, however, significantly increased the apoptosis rate in MDA-MB-231 cells ($p < 0.05$, Supporting Information Fig. S3). Twenty-four and forty-eight hours after irradiation, overexpression of miR-200c or 3-MA treatment or combination with 3-MA and miR-200c mimic significantly increased the rate of apoptosis compared to the scramble negative control group in MDA-MB-231 cells ($p < 0.05$, Supporting Information Fig. S3). In addition, 3-MA dose not synergize with autophagy inhibition induced by miR-200c postirradiation in MDA-MB-231 cells. However, 3-MA significantly increased radiosensitivity in MCF-7 cells after treatment with miR-200c inhibitors (Fig. 3d and Supporting Information Table 4). Also, miR-200c overexpression or 3-MA increased the irradiation-induced activation of caspase-3 and PARP in MDA-MB-231 cells (Fig. 3e). Suppressing miR-200c expression in MCF-7 cells decreased the irradiation-induced activation of PARP (Fig. 3e). Because MCF-7 cells are a caspase-3-deficient breast tumor cell line,32 treatment with miR-200c inhibitors, 3-MA or both, did not affect the expression of activation of caspase-3 postirradiation.

Figure 2. Autophagy is induced by irradiation and acts in a protective manner in breast cancer cells. (a) The breast cancer cell lines MDA-MB-231, BT549 and MCF-7 were exposed to 6 Gy radiation. Autophagosome formation over time was detected via Western blot using antibodies against LC3 and p62. (b) Western blot analysis of autophagy in MDA-MB-231, BT549 and MCF-7 cells following combined treatment with 3-MA (5 mM) and/or irradiation (6 Gy) for 24 hr. (c) The survival fraction of breast cancer cells with irradiation treatment alone or with irradiation and 3-MA (5 mM) treatment.

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However, treatment with 3-MA significantly reversed the decreased activation of PARP proteins induced by miR-200c suppression in MCF-7 cells postirradiation (Fig. 3e). These results suggest that miR-200c enhanced the radiosensitivity of breast cancer cells in a manner that may be associated with the suppression of IR-induced autophagy.

**MiR-200c suppresses irradiation-induced autophagy by targeting UBQLN1**

To investigate the underlying molecular mechanism by which miR-200c suppressed irradiation-induced autophagy, we searched for miR-200c targets using three publicly available databases (TargetScan, Pictar and miRBase). Then, we identified 11 candidate genes that were commonly predicted to be possible targets of miR-200c. Further analysis using Gene Ontology Analysis and manual search of Pubmed highlighted the following four genes related to autophagy: RAC1, NPC1, PPP2CA and UBQLN1. Considering its reported role in autophagosome formation and cancer, we focused on UBQLN1 in our study. Ectopic expression of miR-200c did not affect the mRNA expression level of UBQLN1 (p > 0.05, Fig. 4a), whereas overexpression of miR-200c significantly suppressed the protein expression of UBQLN1 in MDA-MB-231 and BT549 cells (Fig. 4b). In addition, miR-200c decreased the luciferase activity of the wild-type 3’UTR of UBQLN1 but not the mutant reporter gene (Figs. 4c and 4d).

To confirm that miR-200c enhanced radiosensitivity due to the direct targeting of UBQLN1, we examined the effect of silencing the expression of UBQLN1 on radiosensitivity. The clonogenic assay revealed reduced survival fraction by UBQLN1 knockdown in MDA-MB-231 and BT549 cells exposed to irradiation (Fig. 4e, Supporting Information Fig. S4a and Supporting Information Table 5). Silencing UBQLN1 also reduced the cell viability and inhibited irradiation-induced autophagy (Fig. 4f and Supporting Information Fig. S4b). These results demonstrate that miR-200c suppressed irradiation-induced autophagy by directly targeting UBQLN1.
UBQLN1 is a functional miR-200c target

To further investigate the functional relevance of UBQLN1 regulation by miR-200c, we constructed a UBQLN1-expression plasmid in which the UBQLN1 cDNA does not contain the 3′UTR sequence targeted by miR-200c. Co-transfection of UBQLN1 and miR-200c mimics substantially reversed the radiosensitivity of breast cancer cells induced by overexpression of miR-200c (Figs. 5a and 5b and Supporting Information Table 6). Additionally, the restoration of UBQLN1 level enhanced the expression of LC3II and degradation of p62 induced by irradiation (Fig. 5c), which indicated the upregulation of autophagy activity. These results suggest that UBQLN1 is a functional target of miR-200c.

miR-200c, UBQLN1 and autophagy activity are inversely correlated in human breast cancer tissue

Analysis of human breast cancer specimens using ISH and IHC revealed an apparent association of decreased expression of miR-200c with elevated UBQLN1 and LC3 expression, and vice versa. In noncancerous adjacent breast tissues, we detected moderate expression of miR-200c and no expression of either UBQLN1 or LC3 (Figs. 6a and 6b). Neither clinical or pathological characteristics were correlated with the expression of miR-200c, UBQLN1 or LC3 (Supporting Information Table 7). However, lymph node metastasis was inversely correlated to the expression of miR-200c but not UBQLN1 or LC3. Whether the expression of UBQLN1 influences breast cancer metastasis requires further investigation.

Discussion

The results from our study indicated miR-200c as an inhibitor of irradiation-induced autophagy. We also identified UBQLN1 as a functional target of miR-200c in such an action. Ectopic expression of miR-200c enhanced the radiosensitivity of breast cancer cells, which might associate with suppressing autophagy. Our results suggest that miR-200c...
sensitizes breast cancer cells to radiation in a manner associated with the inhibition of irradiation-induced autophagy.

Autophagy is an evolutionarily conserved lysosomal degradation pathway critical for maintaining cellular integrity. Dysregulation of autophagy is implicated in many pathophysiological processes, including neurodegenerative disorders, cardiovascular diseases and cancer. In cancer, autophagy could have two opposing roles. On one hand, autophagy could suppress tumorigenesis. In contrast, autophagy could serve as a prosurvival mechanism when cancer cells are subjected to damage by chemical or physical treatment. Upon irradiation, increased autophagy could protect cancer cells from damage. Consistent with such a notion, the results from our study revealed that autophagy is induced by irradiation and could protect breast cancer cells.

Dysregulation of miRNAs contributes to tumor progression and therapeutic resistance. Previous reports have demonstrated that miR-200c could promote apoptosis and enhance radiosensitivity in cancer cells. In our study, we found that UBQLN1 knockdown could significantly reduce cell viability, enhance the radiosensitivity and suppress irradiation-induced autophagy in breast cancer cells. Restoring UBQLN1 expression reversed the radiosensitivity and radiation-induced autophagy suppression of breast cancer cells induced by overexpression of miR-200c. Such findings may be explained by the previous finding that UBQLN1 could promote autophagosome formation and stabilize BCLb protein and protect cells from apoptotic cell death.

Recent studies have indicated that reducing the expression of UBQLN1 suppresses autophagosome formation, and upregulated UBQLN1 has been associated with poor survival in patients with primary lung adenocarcinomas. In our study, we found that UBQLN1 knockdown could significantly reduce cell viability, enhance the radiosensitivity and suppress irradiation-induced autophagy in breast cancer cells. Restoring UBQLN1 expression reversed the radiosensitivity and radiation-induced autophagy suppression of breast cancer cells induced by overexpression of miR-200c. Such findings may be explained by the previous finding that UBQLN1 could promote autophagosome formation and stabilize BCLb protein and protect cells from apoptotic cell death. Put together, these findings suggest that miR-200c could enhance the radiosensitivity of breast cancer cells by directly targeting UBQLN1.
A recent report indicated an association of decreased miR-200c levels in cancer tissues with advanced clinical stage and lymph node metastasis. Consistent with this report, we detected an inverse correlation between the level of miR-200c expression and lymph node metastasis. However, UBQLN1 or LC3 expression was not correlated with lymph node metastasis. This miR-200c-mediated autophagy pathway was identified as a molecular mechanism that regulates the radioresistance of breast cancer cells and represents a therapeutic target for the treatment of breast cancer.

In conclusion, we demonstrated that downregulation of miR-200c is associated with radioresistance in breast cancer cells, and ectopic expression of miR-200c could inhibit irradiation-induced autophagy and enhance radiosensitivity by directly targeting UBQLN1. This miR-200c/UBQLN1-mediated autophagy pathway was identified as a molecular mechanism that regulates the radioresistance of breast cancer cells and represents a therapeutic target for the treatment of breast cancer.

References

5. Kyndi M, Sorensen FB, Knudsen H, et al. Expression of UBQLN1 in breast cancer and adjacent non-cancerous breast tissues. miR-200c expression was detected via ISH. UBQLN1 and LC3 expression was detected via IHC. The correlation between the expression of miR-200c, UBQLN1 and LC3 in the clinical specimens. The p value was obtained using Fisher’s test. [Color figure can be viewed in the online issue, which is available at wileyonlineibrary.com.]


