Let-7c Governs the Acquisition of Chemo- or Radioresistance and Epithelial-to-Mesenchymal Transition Phenotypes in Docetaxel-Resistant Lung Adenocarcinoma

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Abstract

MicroRNA (miRNA) expression and functions have been reported to contribute to phenotypic features of tumor cells. Although targets and functional roles for many miRNAs have been described in lung adenocarcinoma (LAD), their pathophysiologic roles in phenotypes of chemoresistant LAD cells are still largely unclear. Previously, docetaxel (DTX)-resistant LAD cell lines (SPC-A1/DTX and H1299/DTX) were established by our laboratory and displayed chemo- or radioresistance and mesenchymal features with enhanced invasiveness and motility. Unbiased miRNA profiling indicated that let-7c (MIRLET7C) was significantly downregulated in SPC-A1/DTX cells. Ectopic let-7c expression increased the in vitro and in vivo chemoresistance of DTX-resistant LAD cells through enhanced apoptosis, reversal of epithelial-to-mesenchymal phenotypes, and inhibition of in vivo metastatic potential via inactivation of Akt phosphorylation, whereas a let-7c inhibitor decreased the chemoresistance of parental cells. Further investigation suggested that let-7c significantly reduced the luciferase activity of a Bcl-xL 3'-UTR-based reporter, concordant with reduced Bcl-xL protein levels. Additionally, siRNA-mediated Bcl-xL knockdown mimicked the same effects of let-7c precursor, and enforced Bcl-xL expression partially rescued the effects of let-7c precursor in DTX-resistant LAD cells. Furthermore, we found that Bcl-xL was significantly upregulated in DTX-nonresponding LAD tissues, and its expression was inversely correlated with let-7c expression. This study suggests an important role for let-7c in the molecular etiology of chemoresistant lung adenocarcinoma.

Introduction

Lung adenocarcinoma (LAD) is the most common type of lung cancer, making up 30% to 40% of all non–small cell lung carcinoma (NSCLC) cases (1). Currently, the treatment of advanced LAD may include chemotherapy, radiotherapy, molecular-targeted therapy, or a combination of these (2). However, due to development of resistance to chemo- or radiotherapy, the prognosis of patients with LAD still remains poor. Chemotherapy- or radiotherapy-induced epithelial-to-mesenchymal transition (EMT) in tumor cells has been linked to chemo- or radiotherapeutic resistance (3–5). Therefore, a better understanding of the nature of chemoradiotherapy resistance and the molecular mechanisms that govern chemotherapy-induced EMT in LAD could lead to exploration of novel molecular targets.

miRNAs are a class of noncoding RNAs, which have been reported to be involved in the regulation of many biologic processes including embryonic development and tumorigenesis (6–11). Many miRNAs have been reported to be associated with lung cancer cell survival and proliferation (12). However, there have been few studies focusing on the role of miRNAs in the chemoradioresistance and EMT of lung cancer. Previously, we have identified miRNA expression profiles in docetaxel (DTX)-resistant LAD cell line (SPC-A1/DTX) and found that Let-7c was significantly downregulated (13). Recently, the associations between Let-7c and lung cancer are reported by other research groups. Navarro’ and colleagues reported that members of the lethal-7 (let-7) family were downregulated both in embryonic lung tissue and in lung tumors and low levels of let-7c were associated with absence of metastases and early-stage NSCLC (14). Nagayama and colleagues identified 51 genomic regions associated with non-small cell lung cancer (NSCLC) in a genome-wide association study (15).
with homozygous deletions, which contained 113 genes including 3 miRNA genes (let-7c, hsa-mir-99a, and hsa-mir-125b-2), whose inactivation is possibly involved in lung carcinogenesis (15). These results suggested that Let-7c might play crucial roles in the pathogenesis of lung cancer. However, the associations between Let-7c expression and the chemos- or radiosensitivity and EMT phenotypes of LAD cells are unclear, and the possible molecular mechanisms remain to be further elucidated.

In this study, we comprehensively investigate the biologic functions of Let-7c in chemo- or radioresistance and EMT phenotype of docetaxel-resistant LAD cells. Upregulation of Let-7c could not only restore the sensitivity of docetaxel-resistant LAD cells to chemotherapeutic agents or radiotherapy, but also lead to the reversal of EMT phenotype of docetaxel-resistant LAD cells. Furthermore, B-cell lymphoma-extra large (Bcl-xL) is characterized as a bona fide direct and functional molecular target of Let-7c. Therefore, Let-7c may emerge as an important marker for chemo- or radiotherapy response and metastasis as well as a potential therapeutic target for human LADs.

**Materials and Methods**

**Cell lines**

Two human LAD cell lines (SPC-A1 and NCI-H1299) were purchased from the Tumor Cell Bank of Chinese Academy of Medical Science and cultured in RPMI-1640 medium containing 10% FBS and ampicillin and streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The docetaxel-resistant SPC-A1 cell line (SPC-A1/DTX) was established and preserved in 5.0 μg/L docetaxel as described previously (16). The docetaxel-resistant H1299 cell line (H1299/DTX) was generated and preserved in 20.0 μg/L docetaxel.

**Antibodies and reagents**

The antibodies and reagents are listed in Supplementary Materials and Methods.

**Tissue samples**

A total of 18 LAD tissues were collected from patients with advanced LAD who received chemotherapy at Department of Medical Oncology, Jinling Hospital (Nanjing, PR China) between March 2005 and September 2006. Written permission to use human tumor tissues was obtained from the patients who met all of the following criteria: patients who suffered from primary LAD; a histologic diagnosis of LAD with at least one measurable lesion; a clinical stage of IIIB–IV; first-line chemotherapy with docetaxel 75 mg/m² and cisplatin 100 mg/m² or carboplatin area under curve (AUC) 6 mg/mL administered every 3 weeks for a maximum of 5 cycles; and availability of sufficient tumor tissue for RNA extraction. Tumor response was examined by computed tomography and evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD).

**Construction of plasmid vectors**

To ectopically express Bcl-xL, the Bcl-xL–coding region (367–1,068 nt; Genbank accession no. NM_138578) was subcloned into pcDNA3.1 (Invitrogen) by PCR method using the following primers: sense, 5'-CGCTCGAGATGGTCTCAGAGCAAC-3', reverse, 5'-CGGAATTCTCATTTCCGACTGAAAGTG-3'. To construct a luciferase reporter vector, Bcl-xL 3'-untranslated region (UTR) fragment containing putative binding sites for Let-7c was amplified by PCR using the following primers: sense, 5'-CGTCGAGCAGA-GCTGTTTATGGCC-3', reverse, 5'-GGCGATCCGCCTGGCCAGGCAGG-3' (240 bp) and subcloned into the downstream of luciferase gene in the pLuc luciferase vector (Ambion) and named pLuc-Bcl-xL 3'-UTR-wt. Site-directed mutagenesis of the Let-7c target-site in the pLuc-Bcl-xL 3'-UTR was conducted using the QuiikChange Site-Directed Mutagenesis Kit (Stratagene) and named pLuc-Bcl-xL 3'-UTR-mut, in which Bcl-xL 3'-UTR-wt was used as a template.

**Bioinformatics methods**

The methods of miRNA targets prediction were described in Supplementary Materials and Methods.

**Transfection of oligonucleotides or plasmids and stable selection**

The Let-7c precursor, Let-7c inhibitor (anti-Let-7c), and control miR-NC or anti-miR-NC were synthesized by GenePharm. The plasmids expressing Let-7c or control miR-NC in pSuper.GFP/neo (Invitrogen) were successfully constructed by us and named pSuper/Let-7c or pSuper/miR-NC, respectively. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. All plasmid DNA for transfection was extracted by DNA Midiprep or Midiprep kit (Qiagen). Bcl-xL siRNA (sc-55320 and sc-77361) named Bcl-xL/s1 or Bcl-xL/s2 was purchased from Santa Cruz Biotechnology, Inc. Nonspecific control siRNA (siRNA/NC) was used as control.

**Quantitative real-time PCR assay**

Total cellular or tissue RNA for both mRNA and miRNA analysis was prepared using TRIzol reagent (Invitrogen) and reverse-transcribed according to the manufacturer’s instructions. For mRNA analyses, the first strand of cDNA was synthesized using random primers and Superscript III (Invitrogen) and reverse transcription product was amplified using SYBR Green real-time PCR (ABI). Quantitations were conducted using "Bcl-xL qPCR primers" and "GAPDH qPCR primers" obtained from the Harvard Prim er Bank. For miRNA expression levels, reverse-transcription reaction and real-time PCR (RT-PCR) was conducted using TaqMan miRNA assays (ABI). The expression of Let-7c was normalized using U6 rRNA as an internal control (Applied Biosystems). Both SYBR Green and TaqMan quantitative PCR (qPCR) were conducted using the ABI 7500 fast RT-PCR system and the data were analyzed by software SPSS V13.0.
Western blotting
Standard procedures for Western blotting are described in Supplementary Materials and Methods.

Morphologic analysis
Cells were grown to 70% confluence in the appropriate docetaxel-free media for the parental cell lines and media with docetaxel for the docetaxel-resistant cell lines and visualized via light microscopy. Digital pictures were taken from a camera mounted to the microscope.

Colony formation assay
A total of 1.0 × 10⁵ transfected cells were plated in 10-cm culture dishes. After 14 days, cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted.

Transwell migration and invasion assays
Cell migration and invasion were assessed with modified Boyden chamber (Becton Dickinson Labware) assays as described by Grille and colleagues (17). Briefly, approximately 1.0 × 10⁵ cells were plated into the upper chamber of a polycarbonate Transwell filter chamber (Corning). After 12, 24, and 36 hours, cells that did not migrate were removed from the top side of the inserts with a cotton swab. Cells that had migrated to the underside of the inserts were stained with 4% paraformaldehyde, stained with crystal violet, and the migratory cells were counted under a microscope. Cells were counted in 5 random fields per insert. The invasion assay was done in a similar fashion except that the 8.0-μm pore size membrane inserts were coated with Matrigel. Three independent experiments were carried out.

Luciferase reporter assay
For luciferase reporter assays, HEK293T cells (3.0 × 10⁵) were plated in a 24-well plate and then cotransfected with 100 ng of pLuc-Bcl-xL 3'-UTR-wt or pLuc-Bcl-xL 3'-UTR-mut and 50 nmol/L of Let-7c precursor or control miR-NC using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were harvested and assayed with Dual-Luciferase Reporter Assay kit (Promega) according to the manufacturer’s instructions. Transfections were conducted in duplicates and repeated at least 3 times in independent experiments.

In vitro chemosensitivity assay
Single-cell suspensions were prepared and dispersed in 96-well plates. After incubation for 72 hours with the docetaxel or paclitaxel compounds (Sigma), the MTT (Sigma) solution (0.5 mg/mL) was added. Following incubation for 4 hours, 100 μL of extraction buffer were added to each well. After an overnight incubation, absorbance at 490 nm was measured using a microplate reader (Bio-Rad, Model 680).

In vitro radiosensitivity assay
Detection of radiosensitivity was done using clonogenic survival assay. Briefly, the cells were seeded in triplicate at limiting dilutions in 6-well plates for about 24 hours in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS until attached. Twenty-four hours later, the cells were irradiated with different doses of X-ray radiation (0, 2.0, 4.0, 6.0, and 8.0 Gy), respectively. The medium was replaced with a fresh one 24 hours after irradiation. After 7 days of incubation, the colonies were fixed with methanol, stained with 0.5% crystal violet in absolute ethanol, and colonies with 50 or more cells were counted under dissection microscope. In each irradiation dose group, surviving fraction of cells was calculated as plating efficiency of the irradiated cells divided by the plating efficiency of the mock control.

Flow-cytometric analysis of apoptosis
The protocol is described in Supplementary Materials and Methods.

In vivo chemotherapy assay
Animal studies were conducted according to institutional guidelines. Approximately, 5.0 × 10⁶ SPC-A1/DTX/miR-NC or SPC-A1/DTX/Let-7c cells were suspended in 100 μL PBS and injected subcutaneously into the right side of the posterior flank of female BALB/c athymic nude mice (Department of Comparative Medicine, Jinling Hospital) at 5 to 6 weeks of age. Tumor growth was examined every other day with a vernier caliper. Tumor volumes were calculated by using the equation: V = A × B²/2 (mm³), wherein A is the largest diameter and B is the perpendicular diameter. When the average tumor size reached about 50 mm³, docetaxel was given through intraperitoneal injection with a concentration of 1.0 mg/kg, 1 dose every other day with 3 doses totally. After 5 weeks, all mice were killed, and necropsies were carried out. The primary tumors were excised, paraffin-embedded, formalin-fixed, and conducted hematoxylin and eosin staining, immunostaining analysis for proliferating cell nuclear antigen (PCNA) protein expression according to the manufacturer’s instructions.

In vivo radiotherapy assay
Animal studies were conducted according to institutional guidelines. Approximately, 5.0 × 10⁶ SPC-A1/DTX/miR-NC or SPC-A1/DTX/Let-7c cells were suspended in 100 μL PBS and injected subcutaneously into the right side of the posterior flank of female BALB/c athymic nude mice (Department of Comparative Medicine, Jinling Hospital) at 5 to 6 weeks of age. Tumor growth was examined every other day with a vernier caliper. Tumor volumes were calculated by using the equation: V = A × B²/2 (mm³), wherein A is the largest diameter and B is the perpendicular diameter. When the average tumor size reached about 50 mm³, the tumor-bearing nude mice were exposed to X-ray of 2.0 Gy alone for each time. The same treatment for each group was repeated 3 times (the interval time was 5 days). After 4 weeks, all mice were killed, and necropsies were carried
out. The primary tumors were excised, paraffin-embedded, formalin-fixed, and conducted H&E staining, immunostaining analysis for PCNA protein expression according to the manufacturer’s instructions.

**In vivo tumor metastasis assay**

Animal studies were conducted to determine the effect of Le7-7c on in vivo metastasis of CRC cells using female BALB/c athymic nude mice (Shanghai SLAC Laboratory Animal Co., Ltd.) at 5 to 6 weeks of age. Exponentially growing SPC-A1/DTX/Let-7c or SPC-A1/DTX/miR-NC cells were split and grown in a fresh medium for 1 more day before harvest for inoculation. A total of 0.8 million cells were injected into each mouse through tail veins. After 8 weeks of injection, the animals were killed, and lung or liver tumor nodules were counted. Survival tests were conducted using groups of mice (n = 6/group) treated as above and monitored daily until all the mice died. All animal studies were conducted in accordance with the protocols that were approved by the Jiangsu Province Animal Care and Use Committee.

**Statistical analysis**

Data were presented as mean ± SEM from at least 3 separate experiments. Multiple group comparisons were conducted using ANOVA with a post hoc test for subsequent individual group comparison. A P value of less than 0.05 was considered statistically significant.

**Results**

**Establishment and characterization of docetaxel-resistant LAD cell lines**

Docetaxel-resistant LAD cell lines (SPC-A1/DTX or H1299/DTX) from parental SPC-A1 or H1299 cell line were established in our laboratory. Calculating the relative resistance (as a resistance factor) via the ratio of IC_{50} or ED_{50-resistant variant/IC_{50} or ED_{50} of parental cell line, SPCA-1/DTX cell line was 5.37- or 3.46-fold resistant to docetaxel or paclitaxel (13.58 or 0.61 μg/L) and 2.57-fold resistant to irradiation (2.6 Gy; Fig. 1A). Likewise, compared with parental cell line, H1299/DTX cell line was 3.56- or 2.67-fold resistant to docetaxel or paclitaxel (15.24 or 0.74 μg/L) and 3.88-fold resistant to irradiation (3.3 Gy; Fig. 1B). Also, docetaxel-resistant LAD cell lines were shown to be morphologically distinct from their respective parental cell lines and show loss of cell polarity causing a spindle-cell morphology, increased intercellular separation signifying loss of intercellular adhesion, and increased formation of pseudopodia (Fig. 1C). These changes are typical of cells with a mesenchymal phenotype. Then, quantitative RT-PCR (qRT-PCR) and Western blotting were conducted to detect expression of epithelial adhesion markers (E-cadherin and NCadherin) and mesenchymal markers [vimentin and smooth muscle actin (SMA)]. The relative expression of E-cadherin mRNA and protein in SPC-A1/DTX or H1299/DTX cells was significantly downregulated in comparison with parental SPC-A1 or H1299 cells (P < 0.05), whereas that of N-cadherin, SMA, and vimentin mRNA and protein was significantly upregulated (P < 0.05; Fig. 1D and E).

To determine whether Le7-7c affects the sensitivity of LAD cells to docetaxel or paclitaxel, SPC-A1 (or H1299) or SPC-A1/DTX (or H1299/DTX) cells were transfected with anti-Let-7c (anti-miR-NC) or Let-7c precursor (miR-NC), respectively. Forty-eight hours after transfection, the expression of Let-7c was detected by qRT-PCR assay (Fig. 2A). Compared with miR-NC–transfected cells, the expression of Let-7c in anti-Let-7c–transfected SPC-A1 or H1299 cells was increased by 523% or 465%, respectively (P < 0.01). Compared with anti-miR-NC–transfected cells, the expression of Let-7c in anti-Let-7c–transfected SPC-A1 or H1299 cells was downregulated by 42.8% or 38.7%, respectively (P < 0.05). Colony formation assay (Fig. 2B) showed that Let-7c precursor
could inhibit growth of SPC-A1/DTX and H1299/DTX cells \( (P < 0.01) \), whereas Let-7c inhibitor could increase growth of parental cells \( (P < 0.05) \). Finally, the effect of Let-7c expression on chemosensitivity of LAD cells was determined by detection of IC\(_{50}\) values using MTT assay (Fig. 2C and D). Compared with SPC-A1/anti-miR-NC cells, the IC\(_{50}\) value of docetaxel or paclitaxel in SPC-A1/anti-Let-7c cells was increased by 50.5% or 83.6%, respectively \( (P < 0.05) \). Compared with SPC-A1/DTX/miR-NC cells, the IC\(_{50}\) value of docetaxel or paclitaxel in SPC-A1/DTX/Let-7c cells was reduced by 53.1% or 49.0%, respectively \( (P < 0.05) \). These data showed that down-regulation of Let-7c was linked with docetaxel resistance of LAD cells.

**Expression of Let-7c was correlated with in vitro radiosensitivity of LAD cells**

The effects of irradiation on Let-7c and Bcl-xL expression in LAD cells were analyzed. Twenty-four hours after SPC-A1 and H1299 cells were treated with various doses of irradiation \( (0, 2.0, 4.0, \text{and} 6.0 \text{Gy}) \), the expression of Let-7c and Bcl-xL mRNA or protein was determined by qRT-PCR and Western blotting assays. The expression of Let-7c in SPC-A1 or H1299 cells was decreased sequentially with various doses of irradiation \( (0, 2.0, 4.0, \text{and} 6.0 \text{Gy}) \), whereas the expression of Bcl-xL mRNA and protein was increased. The effects of irradiation on Let-7c and Bcl-xL expression in LAD cells were analyzed. Twenty-four hours after SPC-A1 and H1299 cells were treated with various doses of irradiation \( (0, 2.0, 4.0, \text{and} 6.0 \text{Gy}) \), the expression of Let-7c and Bcl-xL mRNA or protein was determined by qRT-PCR and Western blotting assays. The expression of Let-7c in SPC-A1 or H1299 cells was decreased sequentially with various doses of irradiation \( (0, 2.0, 4.0, \text{and} 6.0 \text{Gy}) \), whereas the expression of Bcl-xL mRNA and protein was increased. The effects of irradiation on Let-7c and Bcl-xL expression in LAD cells were analyzed. Twenty-four hours after SPC-A1 and H1299 cells were treated with various doses of irradiation \( (0, 2.0, 4.0, \text{and} 6.0 \text{Gy}) \), the expression of Let-7c and Bcl-xL mRNA or protein was determined by qRT-PCR and Western blotting assays. The expression of Let-7c in SPC-A1 or H1299 cells was decreased sequentially with various doses of irradiation \( (0, 2.0, 4.0, \text{and} 6.0 \text{Gy}) \), whereas the expression of Bcl-xL mRNA and protein in those cells was increased sequentially with various doses of irradiation (Fig. 3A). Forty-eight hours after SPC-

**Figure 1.** Docetaxel-resistant clones (SPC-A1/DTX or H1299/DTX) were derived from LAD cell lines (SPC-A1 or NCI-H1299), respectively. A, chemoresistance assays in parental and docetaxel-resistant SPC-A1 cells. Compared with SPC-A1, SPC-A1/DTX cell line was approximately 5.37- or 3.46-fold resistant to docetaxel or paclitaxel and 3.88-fold resistant to irradiation. B, chemoresistance assays in parental and resistant H1299 cells. Compared with H1299, H1299/DTX cell line was approximately 3.56- or 2.67-fold resistant to docetaxel or paclitaxel and 3.88-fold resistant to irradiation. C, morphologies of parental and resistant cell lines. Cells were grown to 80% confluence and then photographed under \( \times 10 \) (top) or \( \times 20 \) (bottom) magnification. Bars, 30 \( \mu \)m. D-F, migration and invasion of parental and docetaxel-resistant LAD cells. Cells per \( \times 20 \) high-power field migrating or invading at indicated time points. Each experiment was carried out at least 3 times. **, \( P < 0.05 \); **, \( P < 0.01 \).
A1 (or H1299) or SPC-A1/DTX (or H1299/DTX) cells were transfected with anti-Let-7c or Let-7c precursor and irradiation treatment, the effect of Let-7c expression on colony formation of LAD cells was analyzed. Compared with that of anti-miR-NC–transfected SPC-A1 or H1299 cells combined with irradiation treatment (4.0 Gy), the capacity of colony formation was increased in anti-Let-7c–transfected SPC-A1 or H1299 cells combined with irradiation treatment (Fig. 3B). Compared with that of miR-NC–transfected cells combined with irradiation treatment, the capacity of colony formation was significantly decreased in Let-7c precursor-transfected SPC-A1/DTX or H1299/DTX cells combined with irradiation treatment (4.0 Gy; Fig. 3C). The clonogenic survival assay was conducted to analyze the changes of radiosensitivity by determining the 50% effective dose (ED50; Fig. 3D). Compared with that of anti-miR-NC–transfected cells, the ED50 of anti-Let-7c–transfected SPC-A1 or H1299 was increased by 48.1% or 57.9%, respectively (P < 0.05). Compared with that of miR-NC–transfected cells, the ED50 of Let-7c precursor-transfected SPC-A1/DTX or H1299/DTX cells was reduced by 36.5% or 32.2%, respectively (P < 0.05). Thus, downregulation of Let-7c was correlated with the decreased radiosensitivity of chemoresistant LAD cells.

**Expression of Let-7c was linked with formation of EMT phenotype in docetaxel-resistant LAD cells**

Docetaxel-resistant LAD cells have a fibroblast-like morphology that is typical of mesenchymal phenotype of cells associated with the loss of epithelial markers. However, the role of Let-7c in the formation of EMT phenotype in docetaxel-resistant LAD cells is unclear. Then, we investigated whether EMT phenotype of docetaxel-resistant LAD cells could be reversed by Let-7c precursor. Let-7c precursor-transfected SPC-A1/DTX or H1299/DTX cells
displayed round cell-like morphology at the present of FBS (Fig. 4A). Subsequently, expression of both epithelial and mesenchymal molecular markers was determined by Western blotting, and we showed that the expression of epithelial protein markers (E-cadherin and β-catenin) was significantly increased in Let-7c precursor-transfected SPC-A1/DTX or H1299/DTX cells. In contrast, the mesenchymal markers (N-cadherin, vimentin, and SMA), which are positively correlated with EMT, were dramatically downregulated (Fig. 4B). Finally, we analyzed the effect of Let-7c expression on migration and invasion of docetaxel-resistant LAD cells (Fig. 4C). The Let-7c precursor-transfected SPC-A1/DTX cells were decreased by 14.3%, 39.0%, and 58.1% in migration or 17.2%, 22.3%, and 42.7% in invasion at 12, 24, and 36 hours, respectively, relative to miR-NC–transfected cells. Also, Let-7c precursor-transfected H1299/DTX cells were decreased by 22.1%, 43.5%, and 54.2% in migration or 23.1%, 41.6%, and 59.2% in invasion at 12, 24, and 36 hours, respectively, relative to miR-NC–transfected cells. Thus, downregulated Let-7c might be an important regulator of EMT phenotype of chemoresistant LAD cells.

**Effect of Let-7c expression on in vivo chemo- or radiosensitivity and metastatic potential of LAD cells**

To determine the effect of Let-7c on in vivo chemosensitivity of docetaxel-resistant LAD cells, subcutaneous tumors were generated in nude mice followed by treatment with docetaxel. The tumors formed from SPC-A1/DTX stably transfected with pSuper/Let-7c grew more slowly than those formed from control vector-transfected cells after the treatment with docetaxel at day 35 (Fig. 5A). To confirm this, tumor volume at 35 days after inoculation was measured. The average tumor volume of SPC-A1/DTX/miR-NC or miR-NC–transfected cells plus irradiation (4.0 Gy) was significantly increased compared with the control group. Thus, downregulated Let-7c might be an important regulator of EMT phenotype of chemoresistant LAD cells.

**Figure 3.** Effect of Let-7c expression on radiosensitivity of parental or docetaxel-resistant LAD cells. A, qRT-PCR analysis of Let-7c and Bcl-xL mRNA expression and Western blot analysis of Bcl-xL protein expression in SPC-A1 or H1299 cells in the presence of various doses of irradiation (0.0, 2.0, 4.0, or 6.0 Gy), respectively. B, effect of anti-Let-7c on the colony formation capacity of SPC-A1 or H1299 cells combined with irradiation treatment (4.0 Gy). C, effect of Let-7c precursor on the colony formation capacity of SPC-A1/DTX or H1299/DTX cells combined with irradiation treatment (4.0 Gy). D, determining the ED50 values (irradiation) of SPC-A1 or H1299 cells transfected with miR-NC or Let-7c precursor and SPC-A1/DTX or H1299/DTX cells transfected with anti-miR-NC or anti-Let-7c. Each experiment was carried out at least 3 times. Bcl-xL abundance was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Let-7c expression was normalized to U6. *, P < 0.05; **, P < 0.01.
These results indicated that upregulation of Let-7c could prolong the lifespan of mice bearing SPC-A1/DTX tumor cells (Fig. 5E and F). Next, we analyzed the effect of Let-7c on metastatic potential of docetaxel-resistant LAD cells. SPC-A1/DTX/miR-NC cells formed from SPC-A1/DTX/Let-7c cells showed a significantly decreased average number of lung and liver nodules after 4 weeks of irradiation treatment compared with those in tumors formed from SPC-A1/DTX/miR-NC cells (Fig. 5E). Following the treatment with either docetaxel or irradiation, immunostaining showed that PCNA-positive cells in tumors formed from SPC-A1/DTX/Let-7c cells were significantly decreased compared with those in tumors formed from SPC-A1/DTX/miR-NC cells (Fig. 5E and F). Next, we analyzed the effect of Let-7c on metastatic potential of docetaxel-resistant LAD cells. SPC-A1/DTX/Let-7c cells showed a significant decrease of the average number of lung and liver nodules after 4 weeks of injection compared with SPC-A1/DTX tumor cells (Fig. 5G). Furthermore, we showed that upregulation of Let-7c prolonged the lifespan of mice bearing SPC-A1/DTX tumor cells (P = 0.006; Fig. 5H). These results indicated that upregulation of Let-7c could reduce the in vivo chemo- or radiosensitivity and metastatic potential of docetaxel-resistant LAD cells.

Bcl-xl is a functional target of Let-7c

The molecular mechanisms responsible for the multiple functions of Let-7c in docetaxel-resistant LAD cells were investigated. By analyzing potential target genes of Let-7c using miRNA Target Databases, Bcl-xL was identified as a potential target gene of Let-7c and the potential binding site for Let-7c. The molecular mechanisms responsible for the multiple functions of Let-7c in docetaxel-resistant LAD cells were investigated. By analyzing potential target genes of Let-7c using miRNA Target Databases, Bcl-xL was identified as a potential target gene of Let-7c and the potential binding site for Let-7c.

Figure 4. Effect of Let-7c expression on EMT phenotype of docetaxel-resistant LAD cells. A, Let-7c precursor could reverse EMT phenotypes of docetaxel-resistant SPC-A1/DTX or H1299/DTX cells. Bars, 60 μm (a, b, c, and d) and 60 μm (e, f, g, and h). B, docetaxel-resistant SPC-A1/DTX or H1299/DTX cells transfected with miR-NC or Let-7c precursor were assessed for the expression of markers of epithelial and mesenchymal phenotypes by Western blotting. C, migration and invasion of docetaxel-resistant SPC-A1/DTX or H1299/DTX cells transfected with miR-NC or Let-7c precursor. Cells per ×20 high-power field migrating or invading at indicated time points. Each experiment was carried out at least 3 times. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. ** P < 0.01; *** P < 0.001.
Roles of Bcl-xL in formation of chemo- or radiosensitivity and EMT phenotype in docetaxel-resistant LAD cells induced by downregulation of Let-7c

Bcl-xL has been found to be overexpressed in many human cancers including lung cancer, but the biopathologic significance of Bcl-xL in the formation of chemo- or radiosensitivity and EMT phenotype of docetaxel-resistant LAD cells is still unclear. To explore the biologic functions of Bcl-xL in docetaxel-resistant LAD cells, 2 siRNAs (Bcl-xL/s1 and Bcl-xL/s2) were used to knockdown endogenous Bcl-xL expression. Bcl-xL protein could be downregulated in Bcl-xL/s2–transfected SPC-A1/DTX or H1299/DTX cells but not in Bcl-xL/s1–transfected cells (Fig. 6D), and Bcl-xL downregulation resulted in the decreased colony formation capacity of docetaxel-resistant LAD cells (P < 0.01; Fig. 6E). In addition, Bcl-xL downregulation could decrease the docetaxel (or paclitaxel)-IC50 values of SPC-A1/DTX and H1299/DTX cells, respectively (P < 0.01; Fig. 6E). Also, Bcl-xL downregulation could lead to the decreased irradiation-ED50 values of SPC-A1/DTX or H1299/DTX cells, respectively (P < 0.05; Fig. 6G). The effects of Bcl-xL downregulation on expression of EMT-related molecular markers were then determined. It can be observed that the expression of epithelial protein markers (E-cadherin and
β-catenin) was reduced in Bcl-xL/s2–transfected SPC-A1/DTX or H1299/DTX cells and the expression of mesenchymal markers (N-cadherin, vimentin, and SMA) was increased (Fig. 6H). Also, the Bcl-xL/s2–transfected docetaxel-resistant LAD cells were decreased in migration or invasion at 12, 24, and 36 hours, respectively, relative to

**Figure 6.** Bcl-xL is a direct target of Let-7c. A, putative Let-7c–binding sequence in the 3′-UTR of Bcl-xL mRNA. Mutation was generated on the Bcl-xL 3′-UTR sequence in the complementary site for the seed region of Let-7c, as shown. A human Bcl-xL 3′-UTR fragment containing wild-type or mutant Let-7c–binding sequence was cloned downstream of the luciferase reporter gene. B, analysis of luciferase activity. HEK293 cells were cotransfected with miR-NC, Bcl-xL/s1, or Bcl-xL/s2, Western blotting was used to detect Bcl-xL protein expression. E, analysis of Bcl-xL protein expression in SPC-A1/DTX (or SPC-A1) or H1299/DTX (or H1299) cells was detected by Western blotting. D, 48 hours after SPC-A1/DTX or H1299/DTX cells and the expression of mesenchymal markers (N-cadherin, vimentin, and SMA) was reduced in Bcl-xL/s2–transfected SPC-A1/DTX or H1299/DTX cells. H, docetaxel-resistant LAD cells transfected with Bcl-xL/s2 or siRNA/NC were assessed for the expression of markers of epithelial and mesenchymal phenotypes by Western blotting. I, migration and invasion of docetaxel-resistant LAD cells transfected with Bcl-xL/s2 or siRNA/NC. Cells per ×10 high-power field migrating or invading at indicated time points. Each experiment was carried out at least 3 times. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. **P < 0.01; ***P < 0.001.
siRNA/NC–transfected cells (Fig. 6I). These data indicated that Bcl-xL-s2 could mimic the effects of Let-7c precursor on phenotypes of docetaxel-resistant LAD cells.

To further testify that Bcl-xL was involved in Let-7c–induced phenotypic changes in docetaxel-resistant LAD cells, we then carried out rescue experiments. SPC-A1/DTX or H1299/DTX cells were transfected with Let-7c precursor for 48 hours and followed by transfection with pcDNA/Bcl-xL, and we found that cotransfection of pcDNA/Bcl-xL and Let-7c precursor could rescue the decreased Bcl-xL protein (Supplementary Fig. S2A). Moreover, the cotransfection could partially rescue inhibition of colony formation capacity and the increased chemo- or radiosensitivity of docetaxel-resistant LAD cells induced by Let-7c precursor (Supplementary Fig. S2B and S2C). Furthermore, the resulting overexpression of Bcl-xL could abrogate the inhibition of migration and invasion in docetaxel-resistant LAD cells induced by Let-7c precursor (Supplementary Fig. S2D). Taken together, restoration of Bcl-xL can partially rescue the phenotypic changes in docetaxel-transfected LAD cells induced by Let-7c precursor, further suggesting that Let-7c functions in docetaxel-resistant LAD cells by downregulation of Bcl-xL.

Caspase-3–dependent apoptotic pathway was activated and Akt activation was disrupted by ectopic Let-7c expression through downregulating Bcl-xL.

Then, we analyzed the molecular mechanisms of Let-7c–induced reversal of chemo- or radioresistance and EMT phenotype of docetaxel-resistant LAD cells by targeting Bcl-xL. Flow cytometry was used to detect the apoptosis of SPC-A1/DTX cells transfected with Let-7c precursor or Bcl-xL/s2 alone or combined with docetaxel (or paclitaxel) or irradiation treatment. Compared with those cells transfected with miR-NC alone or combined with chemotherapeutic agents or irradiation treatment, the apoptosis of SPC-A1/DTX cells transfected with Let-7c precursor or Bcl-xL/s2 alone or combined with docetaxel (or paclitaxel) or irradiation treatment was significantly increased (Fig. 7A). Next, the expression of cleaved or total caspase-9, -3, and PARP protein was assessed whether Let-7c would be implicated in Bcl-xL expression, and Akt-related molecular marker proteins was detected (Fig. 7E). We showed that the levels of pAkt protein were progressively declined with increasing concentrations of MK-2206. However, total AKT levels were not affected by treatment with MK-2206. In addition, the epithelial marker proteins (E-cadherin and β-catenin) were progressively declined and the mesenchymal markers (N-cadherin, vimentin, and SMA) were progressively increased with increasing concentrations of MK-2206. These data further indicated that Let-7c might reverse EMT phenotypes of docetaxel-resistant LAD cells via suppressing the activated Akt expression.

Collectively, we suggested the following regulatory signaling: Let-7c inhibited Bcl-xL expression, then it activated the caspase-3–dependent pathway and suppressed Akt phosphorylation, which eventually induced the increased chemo- or radiosensitivity and reversal of EMT phenotype in docetaxel-resistant LAD cells (Fig. 7E).

Upregulation of Bcl-xL expression was inversely correlated with Let-7c expression in docetaxel-resistant LAD tissues.

A total of 18 eligible patients with advanced LAD treated with docetaxel combined with platinum agents were collected. Tumors were divided into 2 groups: responding (CR + PR; n = 10) and nonresponding (SD + PD; n = 8). Compared with that of responding tumors, the relative expression of Let-7c was significantly lower in nonresponding tumors (P < 0.05; Supplementary Fig. S3A). Meanwhile, the relative level of Bcl-xL expression was significantly higher in nonresponding tumors compared with that of responding tumors (P < 0.05; Supplementary Fig. S3B). Then, we assessed whether Let-7c would be implicated in Bcl-xL expression in docetaxel-sensitive or -resistant LAD tissues. A significant inverse correlation between the expression of Let-7c and that of Bcl-xL was observed in the same 18 LAD tissues treated with docetaxel combined with platinum agents (r = -0.899; P < 0.001; Supplementary Fig. S3C).

Discussion

In this study, we showed that the decreased Let-7c was critical for chemo- or radioresistance and chemotherapy-induced EMT phenotype of LAD cells. Also, restoration of Let-7c could reverse chemo- or radioresistance and mesenchymal features in docetaxel-resistant LAD cells via targeting Bcl-xL.

Docetaxel, a microtubule-stabilizing agent, has been used for adjuvant therapy after resection of localized LAD and in combination with radiation for locally advanced LAD (19). However, the therapeutic results in some patients with
advanced NSCLC are unsatisfying as for intrinsic or acquired chemoresistance, and identification of novel molecular targets for chemosensitization of human LAD will, therefore, have enormous clinical applications (20). cDNA microarray analysis showed that Bcl-xL was significantly upregulated in SPC-A1/DTX cells. However, the roles and molecular mechanisms of Bcl-xL overexpression in docetaxel-resistant LAD cells remain to be elucidated. By conducting in silico screening using PicTar, TargetScan, and miRBase, we found that the 3'UTR of Bcl-xL gene contained binding sites for Let-7c with reasonable scores, which was also reported in hepatocellular carcinoma. Increasing evidence has shown that miRNA expression signatures were correlated with the clinical and biologic characteristics of tumors including responses to therapy (21, 22). The establishment of 2 docetaxel-resistant LAD cell models in our laboratory provided foundation for further research on its chemoresistant mechanisms. Previously, we reported that 53 of 470 human miRNAs were differentially expressed between SPC-A1/DTX and parental SPC-A1 cell line (21/C2, 2-fold), suggesting that the dysregulation of miRNAs might lead to the aberrant expression of their target genes such as oncogenes or tumor suppressors. In previous studies, we have reported that miRNA-200b and miR-100 could reverse chemoresistance of docetaxel-resistant human LAD cells by targeting E2F3 and Plk1, respectively (23, 24). As the molecular mechanisms of tumor chemoresistance are very complicated, the roles of other miRNAs involved in docetaxel resistance of LAD cells need to be further investigated. Therefore, here, we will explore whether Let-7c plays a role in the chemoresistance of LAD cells.

Figure 7. Effect of Let-7c precursor or Bcl-xL/s2 on apoptosis and Akt signaling in docetaxel-resistant LAD cells. A, flow-cytometric analysis of apoptosis in docetaxel-resistant SPC-A1/DTX or H1299/DTX cells transfected with Let-7c precursor or Bcl-xL/s2 combined with docetaxel (or paclitaxel) or irradiation at indicated doses. B, Western blot analysis of the activated caspase-3 (c-caspase-3), total caspase-3, activated caspase-9 (c-caspase-9), total caspase-9, activated PARP (c-PARP), total PARP proteins in SPC-A1/DTX or H1299/DTX cells transfected with Let-7c precursor or Bcl-xL/s2. The results from ectopic Let-7c expression were similar with the results from Bcl-xL silence. C, analysis of pAkt and total Akt protein expression in SPC-A1/DTX or H1299/DTX cells transfected with Let-7c precursor (or miR-NC) or anti-Let-7c (or anti-miR-NC). D, analysis of pAkt, total Akt, EMT-related protein (E-cadherin, β-catenin, N-cadherin, SMA, and vimentin) expression in SPC-A1/DTX cells treated with Akt inhibitor (MK-2206) at various doses (0, 50, or 100 nm). E, schematic overview of Let-7c regulatory signaling. The results were obtained from 3 independent experiments. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. *, P < 0.05; **, P < 0.01.
critical roles in chemoresistance of LAD cells by posttranscriptionally regulating Bcl-xL expression.

In this study, docetaxel-resistant LAD cells not only showed the increased chemoradioresistance, but also acquired EMT phenotype. Thus, the establishment of docetaxel-resistant LAD cell models would provide foundation for further research on the mechanisms involved in the formation of chemo- or radioresistance and EMT phenotypes in chemoresistant LAD cells. From miRNA microarray data, downregulation of Let-7c was also found in SPC-A1/DTX cell line and tested in other docetaxel-resistant LAD cell line (H1299/DTX) by qRT-PCR. The let-7 gene was first discovered in the nematode as a key developmental regulator and became one of the first 2 known miRNAs (the other one is lin-4; ref. 25). The mature form of let-7 family members is highly conserved across species, and it encodes an evolutionarily conserved family of 13 homologous miRNAs located in genomic locations frequently deleted in human cancers (26, 27). Upregulation of Let-7c has been reported to inhibit growth of lung cancer and prostate cancer cell lines and reduce metastases of breast cancer cells (28–30). However, the roles of Let-7c in malignant phenotypes of docetaxel-resistant LAD cells remain to be further elucidated. First, SPC-A1 or H1299 cell line was treated with various doses of docetaxel or irradiation, and we found that the level of Let-7c expression in LAD cell line was sequentially decreased and the expression of Bcl-xL mRNA and protein was sequentially increased, implying that downregulated Let-7c and upregulated Bcl-xL might play important roles in formation of malignant phenotypes in docetaxel-resistant LAD cells. Second, Let-7c precursor could significantly reduce colony formation activity of docetaxel-resistant LAD cells. Third, upregulation of Let-7c could increase the in vitro and in vivo sensitivity of docetaxel-resistant LAD cells to chemotherapy or radiotherapy. The docetaxel-resistant LAD cells showed EMT characteristics, and interestingly, ectopic Let-7c expression showed partial reversal of EMT characteristics of docetaxel-resistant LAD cells as documented by the increased expression of E-cadherin, β-catenin and decreased expression of N-cadherin, SMA, and vimentin. Meanwhile, upregulation of Let-7c could significantly inhibit the in vitro invasion and in vivo metastatic potential of docetaxel-resistant LAD cells, suggesting that the reexpression of Let-7c could be useful for the reversal of EMT phenotype to mesenchymal-to-epithelial transition in chemoresistant LAD cells. To the best of our knowledge, this is the first report on the association between dysregulated Let-7c and EMT phenotype of docetaxel-resistant LAD cells.

In our bioinformatics analysis, Bcl-xL was predicted to be a direct target gene of Let-7c. In hepatocellular carcinoma, the let-7 family of miRNAs including Let-7c has been reported to potentiate sorafenib-induced apoptosis by inhibiting Bcl-xL expression (31). Bcl-xL is an antiapoptotic member of the Bcl-2 family comprising a group of structurally related proteins that play a fundamental role in the regulation of the intrinsic pathway by controlling mitochondrial membrane permeability and the release of the proapoptotic factor (32). Overexpression of Bcl-xL has been reported to be correlated with proliferation, metastasis, and chemoradioresistance of tumor cells (33–35). Our results showed that Bcl-xL silence could reverse chemo- or radioresistance of docetaxel-resistant LAD cells by enhancing apoptosis, which might be associated with the activation of caspase-3–dependent signaling. Also, Bcl-xL silence could reverse EMT phenotype and inhibit migration or invasion in docetaxel-resistant LAD cells, which might be correlated with inhibition of Akt phosphorylation. The phenotypes induced by Bcl-xL silence were similar to those induced by Let-7c restoration. More importantly, overexpression of Bcl-xL could partially rescue the phenotypical changes of LAD cells induced by Let-7c precursor, suggesting that Bcl-xL was a functional target gene of Let-7c.

EMT has been reported to play vital roles in the resistance of tumor cells to most conventional therapeutics (36, 37), and the presence of EMT phenotypic cells in LAD could be one of the reasons that patients with LAD are typically drug-resistant, which contributes to high mortality. Therefore, therapeutic benefits such as the restoration of chemosensitivity or suppression of metastasis will be enabled by reversing EMT phenotype. Among the let-7 family members, Let-7d has been reported to function as novel regulator of EMT and chemoresistant property in oral cancer (38). Also, upregulation of miR-200 and let-7 by natural agents could lead to the reversal of EMT in gemcitabine-resistant pancreatic cancer cells (39). In this study, we first reported that upregulation of Let-7c could induce the same EMT-related changes of docetaxel-resistant LAD cells similar to Bcl-xL/s2. In other researches, Bcl-xL can also lead to the activation of Akt signaling, many of the downstream effectors of which are involved in cell autonomous processes including EMT (40). Then, we attempted to investigate whether Let-7c affected the phosphorylation of Akt by downregulating Bcl-xL, which eventually led to the changes of EMT-related proteins. Although our data testify this point, the molecular mechanisms of Bcl-xL overexpression affecting phosphorylation of Akt need to be further elucidated. Also, Bcl-xL was found to be upregulated in docetaxel-responding LAD tissues, and docetaxel-nonresponding LAD tissues with high levels of Let-7c expression tend to express low level of Bcl-xL protein. This, therefore, indicated that downregulation of Let-7c might be responsible for upregulation of Bcl-xL in docetaxel-nonresponding LAD tissues. Of course, further study of a larger case population is necessary to confirm this assertion. Moreover, we need to define whether the molecular mechanism investigated in this study would be functional in other chemoresistant tumor types.

In summary, our results showed that ectopic Let-7c expression could increase the sensitivity of docetaxel-resistant LAD cells to chemotherapeutic agents or irradiation and reverse their EMT phenotype by targeting Bcl-xL, which will provide a strong rationale for its potential use as a therapeutic target to reverse chemo- or radioresistance in human LADs.


Molecular Cancer Research

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