Original Paper

Linc00518 Contributes to Multidrug Resistance Through Regulating the MiR-199a/MRP1 Axis in Breast Cancer

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Key Words

Breast cancer • LncRNA • Linc00518 • MiR-199a • MRP1 • Multidrug resistance

Abstract

Background/Aims: Long non-coding RNAs (LncRNAs) have been validated to be pivotal mediators in multidrug resistance (MDR) of various cancers. This study aims to explore the roles and molecular mechanisms of linc00518 implicated in chemoresistance in breast cancer. **Methods:** Expressions of linc00518, miR-199a and MRP1 were evaluated by RT-qPCR or western blot. IC₅₀ values of adriamycin (ADR), vincristine (VCR) and paclitaxel (PTX) were determined by XTT assays and cell apoptosis was assessed by flow cytometry. Luciferase reporter and RIP assays were employed to detect the interaction of linc00518, miR-199a and MRP-1. **Results:** linc00518 expression increased nearly 2 fold and MRP1 level elevated about 2.5 fold in breast cancer tissues as compared to that in adjacent normal tissues. Also, almost 2 fold upregulation of linc00518 and MRP-1 expressions was observed in MCF-7 cells than in MCF-10A cells. Additionally, linc00518 level was almost 2.5 fold higher and MRP1 level was about 2 fold increased in ADR-resistant MCF-7 cells (MCF-7/ADR) than in parental cell line MCF-7. Linc00518 knockdown enhanced chemosensitivity to ADR, VCR and PTX, and boosted ADR-, VCR- and PTX-induced apoptosis in MCF-7/ADR cells. miR-199a inhibitor conferred chemoresistance to ADR, VCR and PTX in MCF-7/ADR cells, and suppressing miR-199a reversed multi-drug susceptibility induced by linc00518 knockdown. Furthermore, linc00518 could act as a molecular sponge of miR-199a to repress MRP1 expression. MRP1 depletion increased the sensitivity of MCF-7/ADR cells to ADR, VCR and PTX, and this effect was attenuated following miR-199a inhibition or linc00518 overexpression. Also, linc00518 silencing increased ADR-mediated anti-tumor effect *in vivo*. **Conclusions:** linc00518 downregulation reduced MDR by regulating miR-199a/MRP1 axis in breast cancer.

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Introduction

Breast cancer is the most common cancer in women, accounting for 29% in diagnosed new cases and 14% in estimated deaths [1]. Chemotherapy is a relatively effective

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approach for the treatment of human cancers. However, the existence and development of chemoresistance especially multidrug resistance (MDR) has tremendously restricted and hampered the curative effect of drugs for a variety of tumors, including breast cancer [2, 3]. Therefore, it is necessary to investigate the underlying molecular mechanisms of chemoresistance so as to improve the efficiency of chemotherapy for patients with breast cancer.

LncRNAs are a group of non-coding RNA molecules of length longer than 200 nucleotides, while microRNAs are a class of non-coding regulatory transcripts with the length of almost 21 nucleotides. LncRNAs have been reported to be involved in many cellular and genomic processes associated with carcinogenesis and drug resistance/sensitivity [4]. Moreover, the importance of lncRNAs in the resistance of breast cancer to multiple drugs has been discussed. For example, lncRNA-ATB enhanced trastuzumab resistance and invasion-metastasis cascade in breast cancer by competitive binding to miR-200c, up-regulating ZEB1 and ZNF-217 [5]. LncRNA HOTAIR contributed to tamoxifen resistance by promoting estrogen receptor transcriptional activities [6]. Up-regulation of GAS5 alleviated trastuzumab resistance by acting as a molecular sponge of miR-21, leading to the de-repression of its endogenous target phosphatase and tensin homologs (PTEN) in breast cancer [7]. Long intergenic non-protein coding RNA 518 (linc00518), mapped to chromosome 6, has been demonstrated to be markedly up-regulated in melanoma [8] and triple-negative breast cancer (TNBC) [9]. However, its exact roles and molecular mechanisms in breast cancer have not been well featured till now.

Multidrug resistance protein 1 (MRP1), encoded by ABCC1, is a member of the ATP-binding cassette (ABC) transporter superfamily located on chromosome 16p13.1. Previous studies have found that higher MRP1 expression could confer MDR in various cancers including breast cancer [10, 11]. For instance, enforced expression of MRP1 enhanced adriamycin (ADR) chemoresistance of prostate cancer cells [12]. MRP1 inhibition improved chemosensitivity of glioblastoma multiforme cells to vincristine (VCR) and etoposide [13]. Moreover, MRP1 has been demonstrated to be correlated with the reduction of survival rate and worse prognosis in breast cancer [14, 15].

In this study, we showed that the expression levels of linc00518 and MRP1 were up-regulated in breast cancer tissues and cell line (MCF-7). Moreover, linc00518 and MRP1 expression was found to be higher in MDR breast cells (MCF-7/ADR) than in the parental MCF-7 line. In addition, the expression profile of MRP1 mRNA was positively correlated with linc00518 expression in breast cancer tissues. These data indicate an underlying connection between linc00518 and MRP1-mediated MDR. Therefore, we proceeded to demonstrate that linc00518 facilitated MDR in breast cancer by regulating the miR-199a/MRP1 axis.

Materials and Methods

Clinical specimens

Thirty pairs of breast cancer tissue specimens and the adjacent normal tissues were obtained from breast cancer patients (n=30; n (age < 50) =8; n (age ≥ 50) =22) without any neo-adjuvant therapy before surgery at Huaihe Hospital of Henan University. The study was approved by their Ethics Committee and all patients signed an informed consent before carrying out the study. The clinicopathological details for breast cancer samples were provided in Table 1.

Cell lines and culture conditions

Human mammary epithelial cell line MCF-10A and human breast cancer cell line MCF-7 were obtained from American Tissue Culture Collection (ATCC, Manassas, VA, USA). The ADR-resistant variant MCF-7/ADR cell line was established by stepwise screening of MCF-7 cells exposed continuously over 6 months to increasing concentrations of adriamycin (ADR) at a range of 0.5 to 25 µmol/L. The final treatment concentration of ADR, vincristine (VCR) and paclitaxel (PTX) in MCF-7/ADR cell lines was 10 µM, 5 µM and 1 µM, respectively. MCF-10A cells were cultured in Clonetics MEGM™ mammary epithelial cells growth medium

(Lonza Walkersville, Inc. Walkersville, MD, USA) supplemented with 5% horse serum, insulin (10 µg/ml), hEGF (20 ng/ml), hydrocortisone (0.5 µg/ml) and cholera toxin (100 ng/ml). MCF-7/ADR cell line and the parental cell line MCF-7 were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (PAA, Austria). HEK293T cells were grown in DMEM medium (Invitrogen) supplemented with 10% FBS (PAA). ADR, VCR and PTX were purchased from Harveybo Genentech Co., Ltd. (Beijing, China).

Transfection

The siRNA of linc00518 (si-linc00518#1 and si-linc00518#2) and their scrambled control (si-con); the siRNA of MRP1 (si-MRP1#1 and si-MRP1#2) and their scrambled control (si-con); miR-199a mimic and its scrambled control (miR-con); along with miR-199a inhibitor (anti-miR-199a) and its negative control (anti-miR-con) were all purchased from GenePharma Co. Ltd (Suzhou, China).

The full length sequences of linc00518 were amplified by PCR from genomic DNA and subcloned into pcDNA3.1 vector (Invitrogen) to form linc00518 overexpression plasmid (pcDNA-linc00518). Also pcDNA-linc00518-MUT vector with the mutant binding sites of miR-199a was constructed. The primer sequences for linc00518 are AAGGAAAAAAGCGGCCGAGTCCTCAGAGAGTCCTCTCTCTC (forward) and CCGCTCGAGAGCATTGACTGCTTTTAATTT (reverse). All these constructs were transfected into cells by lipofectamine 2000 (Invitrogen) referring to the instructions of the manufacturer.

RT-qPCR assays

Total RNA was obtained from tissues and cells using Trizol reagent (Invitrogen) according to the protocols of the manufacturer. The cDNA was synthesized by random primer and M-MLV reverse transcriptase (Thermo Scientific, Waltham, Massachusetts, USA). Then Power SYBR™ Green PCR Master Mix (Thermo Scientific) was employed to assess the expression levels of linc00518, miR-199a and MRP1 mRNA. The sequences of the qPCR primers were as below: Linc00518, 5'-GTGAAAATCTGGCTACTCGTCCC-3' (forward) and 5'-CTGACTTTTGCCACAGACTCCTG-3' (reverse); GAPDH, 5'-CCACCCATGGCAAATTCATGGCA-3' (forward) and 5'-TCTAGACGGCAGGTCAAGTCCACC-3' (reverse); MRP1, 5'-TGGGACTGGAATGTCACG-3' (forward) and 5'-AGGAATATGCCCCGACTTC-3' (reverse). The expression levels of miR-199a and snRNA U6 were determined by stem-loop RT-PCR. The primers were as follows: miR-199a, 5'-AAGGCGATTGATACGAGTCAGAACAGGTA-3' (stem-loop), 5'-AGAAGGCGATTGATACGAGTCA-3' (forward) and 5'-GGTCTCCCCAGTGTTCAGATA-3' (reverse); U6, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATG-3' (stem-loop), 5'-GTGCAGGGTCCGAGGT-3' (forward) and 5'-CGCTTCGGCAGCACAT-3' (reverse).

Western blot assays

The protein level of MRP-1 was detected by western blot assays. Briefly, the tissues and cells were lysed with pre-chilled RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitor (cocktail, Roche, Basel, Switzerland) to extract whole proteins. Then, equal amounts of proteins were subjected to SDS/PAGE gel and transferred to nitrocellulose (NC) membrane (Pall, New York, USA). After blocking with 5% non-fat milk, the membranes were incubated with primary antibody against MRP-1 or β-actin (Abcam, Cambridge, UK) overnight at 4 °C, followed by probing with HRP-conjugated secondary antibody for 1 h. ECL kit (Millipore Corp. Bedford, MA, USA) was used to detect the specific protein signal.

Table 1. Association between linc00518 expression and clinicopathological features of breast cancer patients. * $P < 0.05$ was considered significantly significant. Patients were categorized into low- and high-expression groups based on the median expression level of linc00518

Characteristics	Group	Total (n=30)	linc00518 expression		P value
			High (n=19)	Low (n=11)	
Age (years)	< 50	8	6	2	0.424
	≥ 50	22	13	9	
Tumor size (cm)	< 3 cm	19	13	6	0.447
	≥ 3 cm	11	6	5	
Her-2	Positive	18	9	9	0.063
	Negative	12	10	2	
ER	Positive	17	11	6	0.858
	Negative	13	8	5	
PR	Positive	20	12	8	0.592
	Negative	10	7	3	
Grade	G1, G2	13	5	8	0.013*
	G3	17	14	3	
Tumor stage (T)	I, II	14	6	8	0.029*
	III	16	13	3	
Lymph nodes metastasis	No	21	12	9	0.282
	Yes	9	7	2	

Luciferase reporter assays

The partial fragments of linc00518 or MRP-1 3'UTR containing miR-199a binding sites were amplified by PCR and inserted into pGL3-control vectors (Promega, Madison, WI, USA), designated as linc00518-WT and MRP-1-3'UTR-WT. Besides, pGL3 luciferase reporter vector with linc00518-MUT or MRP-1-3'UTR-MUT containing mutant miR-199a binding sites were also constructed. Then linc00518-WT or linc00518-MUT reporter was co-transfected with control Renilla luciferase pRL-TK vectors (Promega) and miR-con, miR-199a, anti-miR-con or anti-miR-199a into HEK293T cells. MRP-1-3'UTR-WT (WT) or MRP-1-3'UTR-MUT (MUT) reporter was co-transfected with pRL-TK and miR-con, miR-199a, miR-199a+pcDNA3.1 vector (vector) or miR-199a+pcDNA-linc00518 into HEK293T cells. Then, the luciferase activity in the cells was measured by dual-luciferase reporter assay system (Promega) 48 h after transfection.

Determination of IC_{50} values

Non-transfected or transfected cells were seeded in 96-well plates and incubated overnight. On the second day, cells were treated with gradient concentrations of ADR/VCR/PTX for 48h. Then cell viability was assessed by XTT cell proliferation assay kit (Abnova, Taiwan). Briefly, 10 μ L XTT solution was added into each well for 3 h incubation at 37°C. The absorbance of each well was measured at a wavelength of 450 nm. The IC_{50} values (half maximal inhibitory concentration) of drugs were assessed in accordance with the relative survival curve.

RNA immunoprecipitation (RIP) assays

RIP assays were employed to investigate the potential endogenous interaction between linc00518 and miR-199a using a Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) in accordance with the protocols of the manufacturer. The primary antibodies against Ago2 and IgG were obtained from Abcam (Cambridge, Massachusetts, USA). The extracted RNA without DNA and protein was then subjected to RT-qPCR in order to assess the enrichment degree of linc00518 and miR-199a.

Apoptosis detection

Non-transfected or transfected cells were treated with drugs for 48 h, and then double-stained with PI and annexin V-FITC by Annexin V-FITC/PI Apoptosis Detection kit (Beyotime, Shanghai, China). Following this, flow Cytometer (BD Biosciences, San Jose, CA, USA) was used to detect apoptosis rate of cells.

Caspase 9 activity detection

The caspase 9 activity in cells was detected by Caspase 9 Activity Assay Kit (Beyotime) according to the instructions of the manufacturer. Briefly, the cells were lysed to obtain the supernatant, which was then subjected to treatment with Ac-LEHD-pNA for 1-2 h at 37°C, followed by the measurement of absorbance at 405 nm wavelength.

Subcellular fractionation

The Nuclear/Cytosol Fractionation Kit (Biovision, San Francisco Bay, CA, USA) was used to isolate the nuclear and cytoplasmic fractions. Then the expression patterns of linc00518, GAPDH and U6 in nuclear and cytoplasm fractions respectively was determined by RT-qPCR assays.

Lentivirus production and infection

The shRNA sh-linc00518#1 (CCGGGCTAGATGGAACCTTAGTGAATC-GAGTTCACCTAAGGTTCCATCTAGCTTTT), sh-linc00518#2 (CCGGCACCTCC-AAAGTGACGACTTACTCGAGTAAGTCGTCACCTTTGGAGGTGTTTTT) and its corresponding control sequences (sh-con) were subcloned into pGLV5/H1/GFP/Puro lentivirus plasmid. These plasmids were then co-transfected with PG-P3-RRE, PG-P2-REV and PG-P1-VSVG into 293T cells. At 72 h after transfection, lentiviruses were collected to infect MCF-7 cells. Subsequently, MCF-7 cells were subjected to screening with puromycin for almost 7 days in order to obtain stable lentivirus-transfected cells.

Tumor formation in nude mice

All procedures were carried out in accordance with the National standards for the care and use of laboratory animals with approval of Institutional Committee for Animal Research. BALB/c athymic nude

mice (5-7 weeks old) were purchased from Hubei Research Center of Laboratory Animal (Wuhan, China). Approximately 4×10^6 MCF-7/ADR cells stably transfected with sh-con, sh-linc00518#1 or sh-linc00518#2 were subcutaneously injected into the armpit of nude mice to form xenograft mice. At 8 days after infection, 0.1 ml PBS or ADR (dissolved in 0.1 ml PBS; 15 mg/kg) was administered intravenously into xenograft mice once every 4 days for 24 days. Body weight and tumor volume were measured at 8, 12, 16, 20, 24, 28, and 32 days after the first injection. At 32 days post inoculation, mice were killed and tumor masses were resected for weight measurement, RT-qPCR and western blot assays.

Statistical analysis

The results were presented as mean $\pm$ standard deviation (mean $\pm$ SD) from at least three independent experiments. Student's *t*-test was used to analyze the difference between two groups and one-way ANOVA was employed to detect the difference between more than two groups. IC_{50} value was determined by SPSS software using probit regression analysis. *P* values < 0.05 were regarded as statistically significant.

Results

Linc00518 and MRP1 levels in breast cancer tissues and cell lines

To investigate the potential roles of linc00518 and MRP1 in breast cancer, the expression patterns of linc00518 and MRP1 were firstly evaluated in 30 pairs of breast cancer tissues and adjacent normal tissues. RT-qPCR assays showed that linc00518 and MRP1 were both highly expressed in breast cancer tissues compared with normal tissues (Fig. 1A and 1B). Moreover, the mRNA expression level of MRP1 was positively correlated with linc00518 level in breast cancer tissues (Fig. 1C). Due to the relevance between MRP1 and MDR, we further explored whether linc00518 was associated with the MDR of breast cancer by constructing ADR-resistant MCF-7 cell line (MCF-7/ADR). As shown in Fig. 1D-1F, the IC_{50} values of ADR, VCR and PTX was notably elevated in MCF-7/ADR cell compared with its parental cell line MCF-7, indicating the successful establishment of MCF-7/ADR cell line with MDR, which was subsequently used to study the roles of linc00518 and MRP1 in MDR. Then, the expressions of linc00518 and MRP1 were also detected in human normal breast epithelial cell line (MCF-10A), breast cancer cell line (MCF-7) and ADR-resistant MCF-7 cell line (MCF-7/ADR). The results showed that linc00518 and MRP1 levels were dramatically up-regulated in MCF-7 cells compared with MCF-10A cells. Interestingly, the expression levels of linc00518 and MRP1 were also strikingly enhanced in MCF-7/ADR cells when compared to MCF-7 cells, indicating that linc00518 and MRP1 might play a role in the development of MDR in breast cancer.

Linc00518 knockdown enhanced multi-drug sensitivity of MCF-7/ADR cells

To investigate the effect of linc00518 on MDR of MCF-7/ADR cells, two small interfering RNA of linc00518 (si-linc00518#1 and si-linc00518#2) and the scrambled control siRNA (si-con) were synthesized. As shown in Fig. 2A, transfection of si-linc00518#1 or si-linc00518#2 effectively decreased linc00518 expression in MCF-7/ADR cells. IC_{50} assays of chemotherapeutic drugs also demonstrated that the depletion of linc00518 led to an enhancement in the sensitivity of MCF-7/ADR cells to ADR (Fig. 2B), VCR (Fig. 2C) and PTX (Fig. 2D). As is well known, chemotherapy drugs can induce apoptosis of cancer cells. Therefore, the effect of si-linc00518#1 and si-linc00518#2 on apoptosis was assessed in MCF-7/ADR cells treated with ADR (10 μ M), VCR (5 μ M) or PTX (1 μ M). The results showed that combination of linc00518 knockdown and ADR, VCR or PTX resulted in the significant increase of apoptotic rate (Fig. 2E-2G) and caspase 9 activity (2H-2J) compared with single drug treatment group in MCF-7/ADR cells ($P=0.035$). These results indicated that depletion of linc00518 increased the sensitivity of MCF-7/ADR cells to ADR, VCR and PTX by inducing apoptosis.

Linc00518 suppressed miR-199a expression by direct interaction

Several studies demonstrated that lncRNAs could perform as competing endogenous RNAs (ceRNAs) to regulate miRNA expression and influence miRNA-mediated biological functions. To investigate the underlying molecular mechanisms of linc00518 in regulating

Fig. 1. The expression patterns of linc00518 and MRP1 in breast tissues and cell lines. RT-qPCR assays were used to detect the expression levels of linc00518 (A) and MRP1 mRNA (B) in breast cancer tumor tissues and adjacent normal tissues. (C) The correlation analysis between linc00518 and MRP1 mRNA in the tumor tissues of breast cancer patients. (D-F) The IC_{50} values of ADR, VCR and PTX in MCF-7 and MCF-7/ADR cells were measured by XTT assays. (G) The expression of linc00518 was evaluated by RT-qPCR assays in MCF-10A, MCF-7 and MCF-7/ADR cells. (H and I) The mRNA level (H) and protein level (I) of MRP1 in MCF-10A, MCF-7 and MCF-7/ADR cells were determined by RT-qPCR and western blot assays, respectively. * $P < 0.05$.

Fig. 2. Effects of linc00518 knockdown on drug resistance and drug-mediated apoptosis in MCF-7/ADR cells. (A) The expression level of linc00518 was measured by RT-qPCR assays in MCF-7/ADR cells transfected with si-con, si-linc00518#1 and si-linc00518#2. (B-D) XTT assays were used to determine the IC_{50} value of ADR, VCR and PTX in MCF-7/ADR cells with or without linc00518 knockdown. Cell apoptosis was evaluated by flow cytometry (E-G) and caspase 9 activity assays (H-J) in MCF-7/ADR cells with the combined treatment of siRNA (si-con, si-linc00518#1 or si-linc00518#2) and chemotherapy drugs (ADR, VCR or PTX). * $P < 0.05$.

MDR of MCF-7/ADR cells, the miRcode online prediction website was used to search for potential miRNA targets of linc00518. The predicted target miRNAs of linc00518 were shown in Table 2. The analysis results showed some complementary sites between linc00518 and miR-199a (Fig. 3A). To explore the potential interaction of linc00518 and miR-199a, we firstly assessed the cellular localization of

linc00518 by measuring its expression in the nucleus and cytoplasm of MCF-7/ADR cells. As presented in Fig. 3B, GAPDH mRNA (cytoplasmic control) and linc00518 were predominantly detected in the cytoplasmic region, while nuclear control transcript U6 was exclusively identified in the nuclear fraction. The results indicated that linc00518 mainly resided in the cytoplasm of MCF-7/ADR cells, suggesting the potential for linc00518 to interact with miR-199a. To further validate this prediction, the wide type (WT) and mutant type (MUT) luciferase reporter vectors of linc00518 were constructed, followed by co-transfection with miR-con, miR-199a mimic, anti-miR-con, or anti-miR-199a into HEK293 cells. The results showed that compared with miR-con group, introduction of miR-199a mimic strikingly reduced luciferase gene expression of linc00518-WT reporter vector while having no obvious effect on linc00518-MUT reporter (Fig. 3C). Also, transfection of anti-miR-199a markedly enhanced luciferase activity of linc00518-WT reporter rather than linc00518-MUT reporter (Fig. 3D). To test the functional relevance between linc00518 and miR-199a, RIP assays were carried out in MCF-7/ADR cell extracts using an antibody against Ago2, a key component of RNA-induced silencing complex (RISC). As expected, linc00518 and miR-199a were substantially enriched by Ago2 antibody compared with IgG antibody (Fig. 3E), indicating linc00518 and miR-199a were present in Ago2-containing miRNA nucleoprotein complexes. Next, pcDNA-linc00518 over-expression plasmid containing the putative binding sites and pcDNA-linc00518-MUT over-expression plasmid with mutant binding sites shown in Fig. 3A were constructed. RT-qPCR assay results showed that the transfection of pcDNA-linc00518 and pcDNA-linc00518-MUT plasmid significantly promoted linc00518 expression compared with pcDNA3.1 empty vector (Fig. 3F). However, compared with pcDNA3.1 empty vector, pcDNA-linc00518 markedly inhibited miR-199a expression, while pcDNA-linc00518-MUT had no effect on miR-199a level (Fig. 3G). The effect of linc00518 knockdown

Table 2. The predicted target miRNAs of linc00518

Target miRNA
miR-133
miR-135
miR-138
miR-138
miR-141
miR-143
miR-145
miR-150
miR-199

Fig. 3. Linc00518 suppressed miR-199a expression through direct interaction. (A) The putative binding sites of linc00518 and miR-199a and the mutant sites in linc00518-MUT luciferase reporter vector were presented. (B) The expression patterns of linc00518, GAPDH (cytoplasmic control transcript), U6 (nuclear control transcript) in nucleus and cytoplasm fractions of MCF-7/ADR cells was assessed by RT-qPCR assays. The data was showed as a percentage of GAPDH, U6 and linc00518 levels and total levels for each were normalized to be 100%. (C and D) Luciferase activity was measured in HEK293 cells co-transfected with linc00518-WT or linc00518-MUT and miR-con, miR-199a mimic, anti-miR-con or anti-miR-199a. (E) RIP assays were performed to detect the binding efficiency of linc00518 and miR-199a to Ago2 in MCF-7/ADR cells. MCF-7/ADR cells were transfected with pcDNA3.1 vector, pcDNA-linc00518, mutant type pcDNA-linc00518, scramble control siRNA (si-con), si-linc00518#1 or si-linc00518#2, then RT-qPCR assays were performed to detect the expression patterns of linc00518 (F) and miR-199a (G). (H) The correlation analysis of miR-199a and linc00518 expression levels in 30 cases of breast cancer tissues. *P<0.05.

on miR-199a expression was also detected. The results showed that introduction of si-linc00518#1 or si-linc00518#2 markedly suppressed linc00518 expression (Fig. 3F) and enhanced miR-199a expression (Fig. 3G) compared with negative control si-con. These data showed that linc00518 repressed miR-199a expression through the putative binding sites. Moreover, RT-qPCR assays further confirmed that miR-199a expression was negatively related to linc00518 expression in breast cancer tissues (Fig. 3H). These data suggest that linc00518 could directly interact with miR-199a to suppress its expression.

MiR-199a inhibitor reversed si-linc00518-mediated multi-drug susceptibility in MCF-7/ADR cells

Functional analyses showed that miR-199a inhibitor enhanced multi-drug resistance of MCF-7/ADR cells to ADR, VCR and PTX (Fig. 4A-4C), suppressed apoptosis (Fig. 4D-4F), and lowered caspase 9 activity (Fig. 4G-4I) in MCF-7/ADR cells. Rescue experiments revealed that introduction of anti-miR-199a greatly abated si-linc00518-induced drug sensitivity to ADR, VCR and PTX (Fig. 4A-4C). Moreover, the pro-apoptosis effect triggered by linc00518 knockdown was substantially abrogated by miR-199a inhibitor, displayed as decrease in apoptotic rate (Fig. 4D-4F) and reduction in caspase 9 activity (Fig. 4G-4I) in si-linc00518-transfected cells following the inhibition of miR-199a. These data indicated that linc00518 knockdown induced multi-drug sensitivity by promoting apoptosis through regulation of miR-199a expression in MCF-7/ADR cells. Furthermore, we further confirmed that miR-199a

Fig. 4. miR-199a inhibitor reversed the effect of linc00518 knockdown on drug resistance and apoptosis in MCF-7/ADR cells. MCF-7/ADR cells were transfected with anti-miR-con, anti-miR-199a, si-con, si-linc00518#1, si-linc00518#2, si-linc00518#1+anti-miR-199a, si-linc00518#2+anti-miR-199a. (A-C) Transfected MCF-7/ADR cells were treated with gradient concentration of ADR (0-100 μ M), VCR (0-50 μ M) or PTX (0-25 μ M) for 24 h, followed by the measurement of IC₅₀ values of ADR (A), VCR (B) and PTX (C). (D-I) Transfected MCF-7/ADR cells were exposed to ADR (10 μ M), VCR (5 μ M) and PTX (1 μ M) for 24 h, followed by the detection of apoptotic rate (D-F) and caspase 9 activity (G-I). (J) miR-199a expression level was detected by RT-qPCR assays at 48 h after transfection. *P<0.05.

level was reduced in MCF-7/ADR cells transfected with anti-miR-199a (Fig. 4J). Additionally, the inhibition of miR-199a could reduce the si-linc00518-mediated promotive effect on miR-199a expression (Fig. 4J).

Linc00518 acted as a "sponge" of miR-199a to enhance MRP1 expression

Many studies have reported that miRNAs exert their action by suppressing corresponding target mRNA expression, hence, the TargetScan online website was used to search for the potential target mRNAs of miR-199a. The predicted target genes of miR-199a were shown in Table 3. MRP1 was found to be the potential target of miR-199a (Fig. 5A). To further validated the hypothesis, the WT-MRP1-3'UTR and MUT-MRP1-3'UTR luciferase reporter vectors were constructed. Then, the effect of miR-199a or simultaneous pcDNA-linc00518 and miR-199a on luciferase activity of WT-MRP1-3'UTR or MUT-MRP1-3'UTR luciferase reporter systems was assessed. As presented in Fig. 5B, enforced expression of miR-199a decreased the luciferase activity of WT-MRP1-3'UTR reporter, whereas this effect could be reversed following linc00518 over-expression. However, compared with the respective control group, little change was observed in the luciferase activity of MUT-MRP1-3'UTR reporter after transfection with miR-199a or miR-199a+pcDNA-linc00518. Subsequently, we investigated the effect of linc00518 and miR-199a on MRP1 expression. The results showed that either linc00518 knockdown or miR-199a up-regulation inhibited MRP1 expression compared with the corresponding control group. Moreover, the miR-199a-induced decrease in MRP1 level could be partly abrogated by introduction of linc00518 overexpression plasmid (Fig. 5C). These data suggested linc00518 might act as an endogenous ceRNA of miR-199a to enhance MRP1 expression. Correlation analysis also showed that MRP1 expression was inversely related to miR-199a expression in breast cancer tissues (Fig. 5D).

MRP1 knockdown-induced multi-drug susceptibility was reversed by suppressing miR-199a or overexpressing linc00518

To study the functions of MRP1, MRP1 si-RNAs (si-MRP1#1 and si-MRP1#2) and their scrambled control (si-con) were synthesized. Western blot assays showed that transfection of si-MRP1 notably decreased MRP1 expression compared with the si-con group, however this effect could be abolished by miR-199a inhibitor and pcDNA-linc00518 (Fig. 6A). Functional analysis showed that depletion of MRP1 enhanced the susceptibility of MCF-7/ADR cells to ADR, VCR and PTX, while this effect of si-MRP1 was

Table 3. The predicted target genes of miR-199a

Target genes	Gene name
MRP1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1
ZNF23	zinc finger protein 23
MAP3K11	mitogen-activated protein kinase kinase kinase 11
LIN7C	lin-7 homolog C (C. elegans)
DDR1	discoidin domain receptor tyrosine kinase 1
SHOC2	soc-2 suppressor of clear homolog (C. elegans)
HAPLN1	hyaluronan and proteoglycan link protein 1
ZFP2	ZFP2 zinc finger protein
CELSR1	cadherin, EGF LAG seven-pass G-type receptor 1

Fig. 5. linc00518 act as a endogenous ceRNA of miR-199a to enhance MRP1 expression. (A) The putative binding sequences between miR-199a and MRP1 predicted by TargetScan online website together with the mutant sites of MRP1-3'UTR region in the MUT-MRP1-3'UTR reporter vector. (B) Luciferase activity was detected in the HEK293 cells co-transfected with WT-MRP1-3'UTR or MUT-MRP1-3'UTR and miR-con, miR-199a, miR-199a + pcDNA3.1 vector, or miR-199a + pcDNA3.1-linc00518 48 h after transfection. (C) Western blot assays were used to measure MRP1 protein expression in MCF-7/ADR cells transfected with si-con, si-linc00518#1, si-linc00518#2, miR-con, miR-199a, miR-199a+ pcDNA3.1 vector, miR-199a + pcDNA3.1-linc00518 at 48 h after transfection. (D) The correlation analysis between miR-199a and MRP1 mRNA in breast cancer tissues (n=30). *P<0.05.

partly abrogated by inhibiting miR-199a or up-regulating linc00518 (Fig. 6B-6D). Furthermore, MRP1 knockdown enhanced ADR-, VCR- and PTX-mediated pro-apoptosis effect in MCF-7/ADR cells, which was also dramatically abolished following miR-199a inhibition or linc00518 overexpression (Fig. 6E-6G).

Depletion of linc00518 enhanced ADR-mediated anti-tumor effect *in vivo*

To explore the effect of linc00518 on tumor growth *in vivo*, MCF-7/ADR cells stably transfected with sh-linc00518#1, sh-linc00518#2 or sh-con control were subcutaneously injected into the armpit of nude mice. At 8 days after injection, 0.1 ml PBS or ADR (dissolved in 0.1 ml PBS; 15 mg/kg) was administered intravenously into the xenograft mice once every 4 days for 24 days. As shown in Fig. 7A, there was no difference in the total weight of mice between different groups. However, a significant decline was observed in volume and weight of tumors derived from sh-linc00518#1- or sh-linc00518#1-transfected MCF-7/ADR cells compared with the sh-con group (Fig. 7B and 7C) ($P=0.028$). Moreover, knockdown of linc00518 boosted ADR-mediated anti-tumor effect (Fig. 7C and 7D). Subsequently, the expression of linc00518 was measured with the results showing that transfection of sh-linc00518#1 or sh-linc00518#1 attenuated linc00518 expression compared with the sh-con group in resected tumor tissues (Fig. 7D). Furthermore, MRP1 expression at mRNA (Fig. 7E) and protein levels (Fig. 7F) were significantly reduced in excised tumor masses with linc00518#1 or linc00518#1 knockdown compared with the sh-con group. All these data suggest that the inhibitory effect of ADR on tumor growth was increased following linc00518 knockdown.

Discussion

In recent years, emerging evidence indicates that lncRNAs are closely correlated with cancer drug resistance/sensitivity [16]. For instance, Bian *et al.* reported that lncRNA

Fig. 6. Anti-miR-199a and pcDNA-linc00518 reversed the promotion effect of MRP1 knockdown on multi-drug susceptibility and apoptosis of MCF-7/ADR cells. MCF-7/ADR cells were transfected with si-con, si-MRP1#1, si-MRP1#2, si-MRP1#1+anti-miR-199a, si-MRP1#2+anti-miR-199a, si-MRP1#1+pcDNA-linc00518, si-MRP1#2+pcDNA-linc00518. (A) MRP1 protein levels measured using western blot assays in MCF-7/ADR cells 48 h after transfection. Transfected MCF-7/ADR cells were treated with gradient concentrations of drugs for 24 h, followed by the determination of IC_{50} values of ADR (B), VCR (C) and PTX (D). (E-G) Transfected MCF-7/ADR cells were treated with ADR (10 μ M), VCR (5 μ M) and PTX (1 μ M) for 24 h, followed by the detection of apoptotic rate using flow cytometry. * $P<0.05$.

Fig. 7. Depletion of linc00518 enhanced ADR-mediated anti-tumor effect in vivo. MCF-7/ADR cells stably transfected with sh-con, sh-linc00518#1 or sh-linc00518#2 were subcutaneously injected into nude mice to form xenograft. At 8 days after infection, 0.1 ml PBS or ADR was administered intravenously into xenograft mice (once every 4 days) for another 24 days. Body weight (A) and tumor volume (B) were measured at 8, 12, 16, 20, 24, 28, 32 days after the first injection. (C) Tumor weight was detected at 32 days after the first injection. (D) RT-qPCR assays were used to test the expression levels of linc00518 in excised tumors of mice with sh-con, sh-linc00518#1 and sh-linc00518#2 transfection. (E and F) RT-qPCR and western blot assays were respectively applied to measure the mRNA (E) and protein (F) expression of MRP1 in removed tumor tissues with or without linc00518 knockdown. * $P < 0.05$.

urothelial carcinoma associated 1 (UCA1) improved resistance of colorectal cancer cells to 5-FU by regulating miR-204-5p/CREB1 axis [17]. Luo *et al.* found that lncRNA EIF3J-AS1 increased multi-drug resistance by inducing autophagy in gastric cancer via regulation of the miR-188-3p/ATG14 axis [18]. In this study, we found that the expression levels of linc00518 and MRP1 were markedly up-regulated in breast cancer tissues, breast cancer cells along with ADR-resistant breast cancer cells (MCF-7/ADR). Also, the expression level of MRP1 mRNA was positively correlated with linc00518 expression in breast cancer tissues. All these data indicated that linc00518 might participate in mediating MDR of breast cancer cells by MRP1. Hence, the roles and molecular mechanisms of linc00518 in MDR were further explored.

Results showed that knockdown of linc00518 by si-linc00518 boosted chemosensitivity of MCF-7/ADR cells to ADR, VCR and PTX. Moreover, transfection of si-linc00518 enhanced ADR-, VCR- and PTX-induced apoptosis.

Subsequently, we proceeded to explore the molecular mechanism of linc00518 in mediating MDR of MCF-7/ADR cells. In the past decades, mounting evidences indicated that lncRNAs could exert their functions by regulating miRNA expression. Previous findings also suggested that multiple miRNAs were implicated in the regulation of drug resistance in breast cancer [19]. For example, miR-298 down-regulation increased ADR chemoresistance of metastatic breast cancer cells [20], whereas over-expression of miR-451 induced the sensitivity of breast cancer cells to ADR [21]. In the present study, we demonstrated that linc00518 could inhibit miR-199a expression by direct interaction and miR-199a expression was negatively related with linc00518 expression in breast cancer tissues.

MiR-199a has been reported to be a tumor suppressor in hepatocellular and testicular cancers as well as breast cancer with a low-level expression [22-25]. Moreover, previous studies have shown that miR-199a markedly enhanced chemosensitivity of ovarian cancer-initiating cells to ADR, PTX and CDDP [26]. Furthermore, miRNAs profiling computation analysis suggested that miR-199a might participate in the regulation of ADR resistance in breast cancer cells [27]. Hence, the role and underlying molecular mechanism of miR-199a in regulating multi-drug resistance of breast cancer cells were further explored. Functional analyses revealed that inhibition of miR-199a enhanced chemoresistance of MCF-7/ADR cells to ADR, VCR and PTX, and attenuated the drug-induced apoptosis and caspase 9 activity. Also, inhibition of miR-199a notably abrogated the effect of linc00518 knockdown on drug resistance and apoptosis in MCF-7/ADR cells.

Many studies have shown that miRNAs exert the functions by inhibiting target mRNA expression. In this study, we demonstrated that MRP1 was a target of miR-199a, which was in accordance with previous studies [28]. Moreover, linc00518 could perform as a ceRNA of miR-199a to enhance target gene MRP1 expression. Some findings showed that miRNAs could mediate drug resistance by targeting MRP1. For instance, miR-7 and miR-345 was involved in mediating CDDP resistance of breast cancer by regulating MRP1 expression [29]. Our study further confirmed that knockdown of MRP1 decreased resistance of MCF-7/ADR cells to ADR, VCR, PTX, while this effect could be abolished by introduction of miR-199a inhibitor and linc00518 over-expression plasmid. Additionally, depletion of MRP1 promoted ADR-, VCR- and PTX-induced apoptosis. Furthermore, the effect of si-MRP1 on apoptosis could be attenuated by transfection of anti-miR-199a and pcDNA-linc00518. Further *in vivo* assay results showed that knockdown of linc00518 blocked tumor growth and exacerbated ADR-mediated anti-tumor effect.

Previous studies indicated that MRP1 was involved in regulating chemoresistance in other breast cancer cell lines (such as triple-negative breast cancer cell line MDA-MB-231) [30-32] besides Luminal A subtype (MCF-7, T47D) [33, 34]. For instance, IL-6/HIF-1 α contributed to chemoresistance of MDA-MB-231 cells by regulating MRP1 expression [30]. Our results showed that MRP1 was a downstream target of linc00518/miR-199 in MCF-7 based cells. Collectively, these data indicated that linc00518/miR-199/MRP1-mediating chemoresistance is not limited to Luminal A subtype of breast cancer.

Collectively, our present study demonstrated that linc00518 increased resistance of MCF-7/ADR cells to ADR, VCR, PTX by reducing ADR-, VCR- and PTX-induced apoptosis through the miR-199a/MRP1 axis. These findings may provide further valuable insight into the regulatory mechanisms of lncRNA in MDR and highlight new promising strategies to relieve MDR resistance.

Disclosure Statement

The authors have no conflict of interests to declare.

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