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Long noncoding RNA UCA1 modulates breast cancer cell growth and apoptosis through decreasing tumor suppressive miR-143

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Abstract. – OBJECTIVE: Long non coding RNA (LncRNA) urothelial carcinoma-associated 1 (UCA1) is an oncogene in breast cancer. However, the detailed mechanism has not been fully revealed. This study explored whether UCA1 can directly interact with miR-143, a tumor suppressor in breast cancer and whether the UCA1-miR-143 axis is involved in regulation of cancer cell growth and apoptosis.

PATIENTS AND METHODS: miRNA microarray was performed to identify the most dysregulated miRNAs between tumor and adjacent normal tissues of breast cancer. QRT-PCR analysis was performed to assess the expression of UCA1 and miR-143. The binding between UCA1 and miR-143 was verified using dual luciferase and RNA binding protein immunoprecipitation (RIP) assay. MTT assay and flow cytometry analysis were performed to study the role of UCA1-miR-143 axis in cell proliferation, cell cycle and apoptosis.

RESULTS: UC1 was significantly upregulated, while miR-143 was significantly downregulated in the tumor tissues than in the adjacent normal tissues. There are direct interactions between miR-143 and the miRNA recognition sites of UCA1. UCA1 is present in Ago2-containing RNA-induced silencing complex (RISC), through association with miR-143. Through downregulating miR-143, UCA1 can modulate breast cancer cell growth and apoptosis.

CONCLUSIONS: UCA1 can directly interact with miR-143, lower its expression and affect its downstream regulation. Therefore, the UCA1-miR-143 axis constitutes a part of the oncogenic role of UCA1 in breast cancer.

Key Words:
UCA1, miR-143a, Breast cancer, ceRNA.

Introduction

Breast cancer is the most common female malignancy and the second leading mortality of all cancers in women[1]. Although recent target treatment has remarkably improved patient survival, tumor invasion and metastasis is still a major obstacle of breast cancer therapy[2]. Therefore, it is necessary to investigate the molecular mechanism of tumor development, which provides fundamental information for future new therapy.

There are emerging studies showed that long noncoding RNAs (lncRNAs) play an important role not only in normal development but also in tumorigenesis[3]. In fact, a series of studies reported that some lncRNAs are dysregulated and take part in pathological development of breast cancer through multiple mechanisms. For example, lncRNA SPRY4-IT1 can promote the proliferation of human breast cancer cells via upregulating ZNF703 expression[4], which is a common Luminal B breast cancer oncogene[5]. NF-KappaB Interacting LncRNA (NKILA) interacts with NF-kappaB/IkappaB to form a stable complex, blocking IkappaB phosphorylation and suppressing breast cancer metastasis[6].

Besides the regulation through direct interaction with the target gene, the concept of competing endogenous RNAs (ceRNAs) was proposed to explain a novel regulatory mechanism of RNA. According to this concept, RNAs can cross-talk with each other by competing shared for miRNAs, imposing another level of posttranscriptional regulation[7]. In fact, this mechanism was seen in breast cancer. For example, LincRNA-ROR can prevent degradation of miR-205 target genes, including the epithelial-to-mesenchymal transition (EMT) inducer ZEB2 through sponging miR-205, thereby promoting breast cancer tumorigenesis and metastasis[8]. LncRNA ATB can increase trastuzumab resistance and invasion-metastasis cascade in breast cancer via competitively biding miR-200c, leading to subsequently upregulation of ZEB1 and

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ZNF-217, which induce EMT[9]. Urothelial carcinoma-associated 1 (UCA1) is an lncRNA firstly identified in bladder transitional cell carcinoma[10]. Till now, it was found UCA1 has 1.4kb, 2.2kb and 2.7kb isoforms in different cells, among which the 1.4 kb isoform is contained in the 2.2 kb isoform[11]. In breast cancer, one recent study found the 1.4 kb isoform has an oncogenic role by forming a complex with heterogeneous nuclear ribonucleoprotein I (hnRNP I), leading to downregulated p27 expression[12].

miRNAs also play important roles in breast tumorigenesis[13,14]. miR-143 is generally considered as a tumor suppressor in breast cancer[15,16]. However, how it is downregulated in breast cancer is not clear. In the current study, we examined whether there are direct interactions between the UCA1 and miR-143 and how they are involved in breast cancer growth and apoptosis.

Patients and Methods

Tissue Collection

This study was approved by the Ethics Committee of the Sichuan Provincial People's Hospital, China. Breast cancer tissues and adjacent normal tissues were obtained from 20 breast cancer patients who received surgical resection of between 2013 and 2014 in the hospital. All of the patients had not received local or systemic treatment before the surgery. Informed consent was obtained from the patient before the study. The pathological stages of the tumors were assessed by an experienced pathologist without authorship to this study. Staging was assessed according to the International Union Against Cancer's (UIAC) tumor-node-metastasis (TNM) system. The non-tumorous tissues refer to tissues without obvious tumor cells and at least 3 cm from the edge of the tumor. Cancer tissues had been confirmed by Hematoxylin-Eosin staining and immunohistochemical detection for ER, PR and HER-2, respectively. 4 pairs of tumor tissue and the corresponding adjacent normal tissues were used for miRNA microarray to identify miRNAs expression.

Cell Culture

HEK 293T cells and breast cancer cell line MDA-MB-231 were obtained from ATCC (Manassas, VA, USA). The cancer cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium and HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin. Both cell lines were cultured in an incubator with humidified atmosphere and 5% CO_2 at 37°C.

Plasmid Preparation

The entire UCA1 (1.4 kb isoform) was amplified using RT-PCR using Reverse Transcription System (A3500, Promega, Madison, WI, USA) with the following primers: forward, CGCGGATCCTTTATCAGGCATATTAGCTTTAA; reverse GCGAATTCTGACATTCTTCTGGACAATG. The sequence was cloned into the BamHI and EcoRI sites (underlined in the primers) of the expression vector pCDH-CMV-MCS-EF1-copGFP (System Biosciences, Mountain View, CA, USA) using a Cold Fusion kit (System Biosciences, Mountain View, CA, USA). To produce lentiviral particles for transfection, the expression particles are cotransfected with pPACKH1 Lentivector Packaging Kit (System Biosciences, Mountain View, CA, USA) to HEK 293T cells according to manufacturer's instruction.

To produce luciferase reporter vectors, the entire UCA1 sequence (widetype) was inserted into the downstream of the luciferase gene of pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) and assigned as pmirGLO-UCA1-WT. The mutant reporter without the miR-143 binding sites was generated using QuikChange Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The insertion and mutation were verified using sequencing.

Cell Transfection

UCA1 siRNAs, miR-143 mimics and the corresponding negative controls were purchased from RiBoBio (Shanghai, China). MDA-MB-231 cells were transfected with 50 nM miR-143 mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and transfected with 100 nM UCA1 siRNAs using Hiperfect transfection reagent (QIAGEN, GmbH, Hilden, Germany) according to manufacturer's instruction.

miRNA Microarray and qRT-PCR Analysis

Total miRNAs in the tumor and adjacent normal tissues and in the cell samples were extracted using the miRVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to manufacturer's instruction. Then, total miRNA sam-

ples of 4 randomly selected pairs of tumor tissue and the corresponding adjacent normal tissue were submitted to Shanghai Biotechnology Corporation for array hybridization on an Agilent Human miRNA array (v.12.0). The miRNAs samples were labeled using fluorescein (Cy3 or Cy5) and then used for hybridization on the Affymetrix miRNA Chip. Background subtraction and normalization were performed. The miRNAs with at least 2-fold difference between the paired tissues were candidates for further analysis ($p < 0.05$).

Total RNA in tissue and cell samples were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. cDNA was reversely transcribed using the PrimeScript® RT reagent kit (TaKaRa, Dalian, Liaoning, China). The UCA1 expression level was quantified using the following primers: forward: 5'-TTTGCCAGCCTCAGCTTAAT-3'; reverse: 5'-TTGTCCCCATTTTCCATCAT-3' and SYBR® Premix DimerEraser kit (TaKaRa, Dalian, Liaoning, China) in an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). GAPDH was used as the endogenous control gene. MiR-143 expression was quantified using TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA, USA), with U6 snRNA used as the endogenous control. The results of qRT-PCR analysis were presented using $2^{- CT}$ method.

RNA Binding Protein Immunoprecipitation (RIP) Assay

RIP assay was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) following the manufacturer's protocol. Generally, MDA-MB-231 cells at 80-90% confluency were collected and were lysed using RIP lysis buffer. Then 100 µl cell extract was incubated with RIP buffer containing magnetic beads conjugated with human anti-Ago2 antibody or negative control normal mouse IgG. The samples were incubated with Proteinase K to digest the protein and then the immunoprecipitated RNA was isolated. The purified RNA was further used for qRT-PCR analysis of UCA1 and miR-143.

Dual Luciferase Assay

Human HEK 293T cells (2.0×10^4) were plated in a 24-well plate. Cells were cotransfected with 200 ng of pmirGLO-UCA1-WT or pmirGLO-UCA1-MUT and 50 nM miR-143 mimics using Lipofectamie 2000 (Invitrogen, Carlsbad, CA, USA). 24h after transfection, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to manufacturer's protocol. Firefly luciferase activity was normalized to that of Renilla luciferase.

MTT Assay of Cell Viability

MDA-MB-231 cells after different treatments were plated at 1.5×10^3 cells/well in 96-well plates. Cells were cultured for 4 days and then cell viability was measured by MTT (Sigma-Aldrich, St Louis, MO, USA) assay according to manufacturer's instruction. Absorbance at 490 nm was read by using a spectrophotometric plate reader. Each test was performed in triplicate.

Flow Cytometry Analysis of Cell Cycle DisTribution and Cell Apoptosis

48h after treatment, to assess the proportion of cells in different phases of cell cycle, cells were stained with 20 µg/mL PI (Sigma-Aldrich, St Louis, MO, USA) and 100 µg/mL RNase A in PBS for 15 min at room temperature. To determine the proportion of cells with active caspase 3, cells were stained using Fluorescein Active Caspase-3 Staining Kit (ab65613, Abcam, Cambridge, MA, USA) according to manufacturers' instructions. DNA content and the proportion of cells with active caspase-3 were analyzed using a FACSCaliber (BD Biosciences, Franklin Lakes, NJ, USA). Data acquisition was done using CellQuest 3.2 software (BD Biosciences, Franklin Lakes, NJ, USA). Each test was performed with at least three repeats.

Results

miR-143 is Significantly Downregulated, While UCA1 is Significantly Upregulated in Breast Cancer

The basic characteristics of the patients recruited were summarized in Table I. miRNAs microarray chip was used to measure miRNAs expression in 4 breast cancer tissues and adjacent normal tissues. The six most up-regulated and six most downregulated miRNAs in the paired samples were given in Figure 1A. It is evident that miR-143 is significantly downregulated in the tumor tissues. By performing qRT-PCR analysis in all the 20 tumor and adjacent normal tissues, we

Table I. Patients' characteristics.

	Parameters	Case No.	%
Age (years)	≥ 50	12	60%
	< 50	8	40%
TNM Stage	I-II	14	70%
	III-IV	6	30%
Grade	I	7	35%
	III	8	40%
	III	5	25%
Subtype	Luminal A	6	30%
	Luminal B	10	50%
	Her-2 overexpression	2	10%
	TNBC	2	10%

further verified significantly lower miR-143 expression in the tumor tissues (Figure 2B). Interestingly, based on our preliminary screening of dysregulated lncRNAs in breast cancer, we found inverse expression between UCA1 and miR-143. This lncRNA was significantly upregulated in the tumor tissues than in the adjacent normal tissues (Figure 3C).

UCA1 Directly Interacts with miR-143 and Regulates its Expression in MDA-MB-231 Cells

Since the inverse expression trend between UCA1 and miR-143 was observed, we further studied whether there are direct interactions between them. Through prediction in online databases (miRcode, http://www.mircode.org/), it was observed that there are four putative binding sites between UCA1 and miR-143 (Figure 2A). To verify the influence of UCA1 on miR-143 expression, MDA-MB-231 cells were firstly transfected with si-UCA1 (Figure 2B) or UCA1 lentiviral particles (Figure 2D) respectively. The cells with UCA1 knockdown had significantly increased miR-143 expression (Figure 2C). In contrast, the cells with UCA1 overexpression had significantly decreased miR-143 expression (Figure 2E). To study whether there are direct interactions between UCA1 and miR-143, the UCA1 cDNA was cloned into the luciferase gene and named pmirGLO-UCA1-WT. The mutant construct without predicting binding sites was also

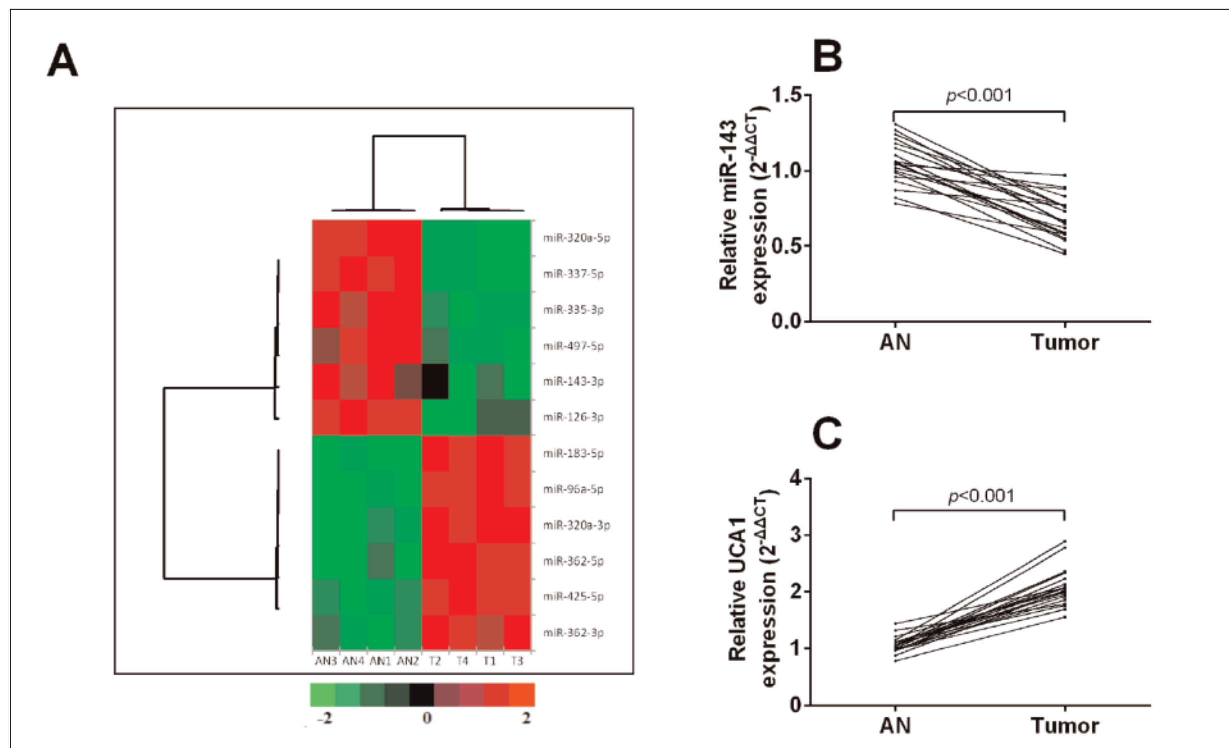

Figure 1. miR-143 is significantly downregulated, while UCA1 is significantly increased in breast cancer. ***A,*** A total of 12 differential expressed miRNAs, including 6 up-regulated and 6 down-regulated, were identified in 4 breast cancer tissues and adjacent normal tissues to the criteria of fold change ≥ 2, $p < 0.05$. Columns represent samples and rows represent miRNAs (black, green, and red correspond to unchanged, down-regulated and upregulated, respectively). T: tumor; AN: adjacent normal. ***B,*** and ***C,*** QRT-PCR analysis of relative miR-143 (B) and UCA1 ***(C)*** in 20 paired breast cancer tumor and adjacent normal tissues.

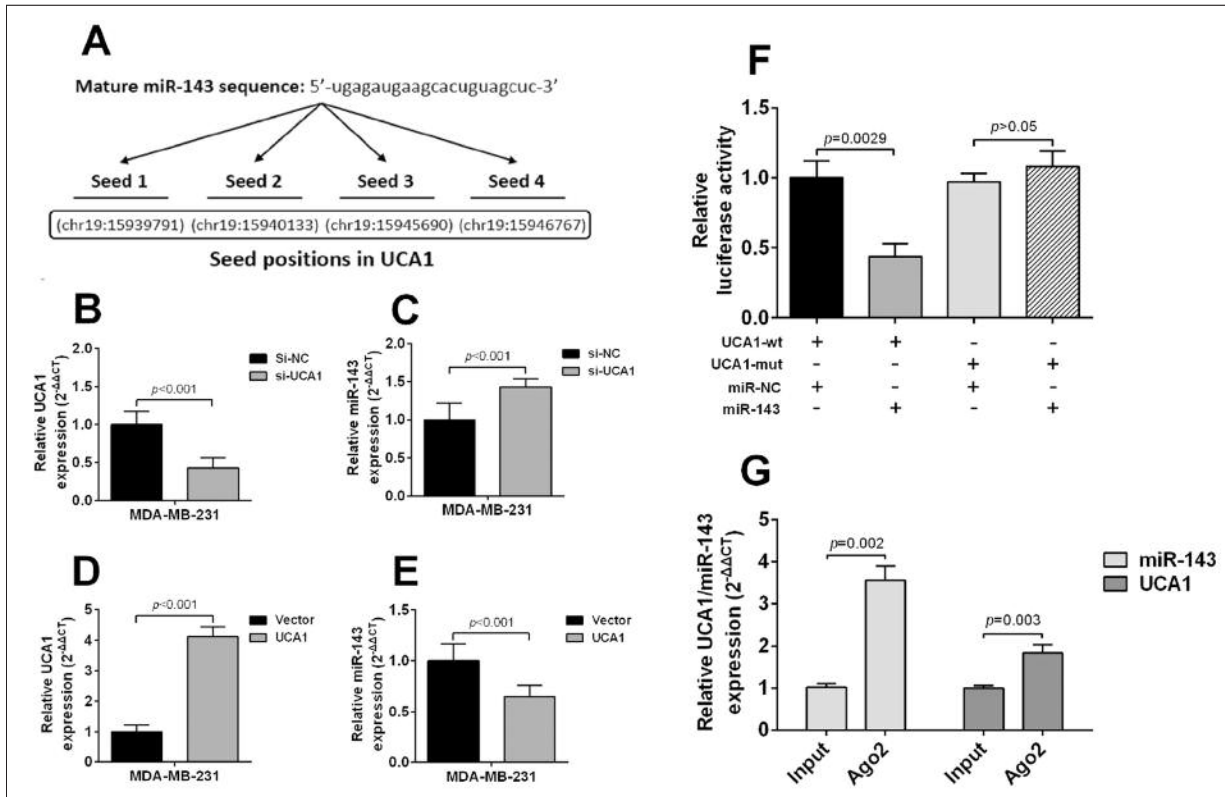

Figure 2. UCA1 can directly interact with miR-143 and regulate its expression in MDA-MB-231 cells. ***A,*** Predict binding sites between UCA1 and miR-143. ***B*** and ***C,*** QRT-PCR analysis of relative UCA1 (B) and miR-143 ***(C)*** in MDA-MB-231 cells transfected with 100 nM si-UCA1. ***D*** and ***E*** QRT-PCR analysis of relative UCA1 (D) and miR-143 ***(E)*** in MDA-MB-231 cells infected with UCA1 lentiviral particles. ***F,*** MDA-MB-231 cells were co-transfected with either 50 nM miR-143 mimics or NC oligos and 200 ng of pmirGLO-UCA1-WT or pmirGLO-UCA1-MUT (wide) using Lipofectamie 2000. The relative firefly luciferase activity measured 24 hours after transfection and was normalized with renilla luciferase activity. ***G,*** Association of UCA1 and miR-143 with Ago2. Cellular lysates from MDA-MB-231 cells were used for RIP assay with Ago2 antibody. UCA1 and miR-143 level was detected using qRT-PCR.

generated. The plasmids were individually transfected into HEK 293T cells together with miR-143 mimics or the negative controls. The results showed that luciferase activity of pmirGLO-UCA1-WT was significantly reduced by miR-143 mimics (Figure 2F). However, this miRNA mimics had no influence on the luciferase activity of pmirGLO-UCA1-MUT (Figure 2F). These results suggested that there are direct interactions between miR-143 and the miRNA recognition sites of UCA1. Since miRNAs exert their gene silencing functions through RNA-induced silencing complex (RISC) containing Ago2[17,18], we hypothesized that UCA1 and miR-143 might be in the same RISC complex. RNA binding protein immunoprecipitation (RIP) assay was performed using MDA-MB-231 cell extracts using antibodies against Ago2. RNA levels in immunoprecipitates were determined by qRT-PCR. The results showed that both miR-143 and UCA1 were significantly enriched in the Ago2 pellet relative to input control (Figure 2G).

UCA1 Modulates Breast Cancer Cell Growth and Apoptosis at Least Partially Through miR-143

Since miR-143 has suppressing effect over cell proliferation and migration in several types of cancer[19,20], including breast cancer[16], we then investigated the role of the UCA1-miR-143 axis in growth and apoptosis of the cancer cells. MTT assay showed that UCA1 and anti-miR-143 significantly increased, while si-UCA1 and miR-143 significantly decreased cell viability of MDA-MB-231 cells (Figure 3 A and B). However, to the cells with UCA1 overexpression, trans-

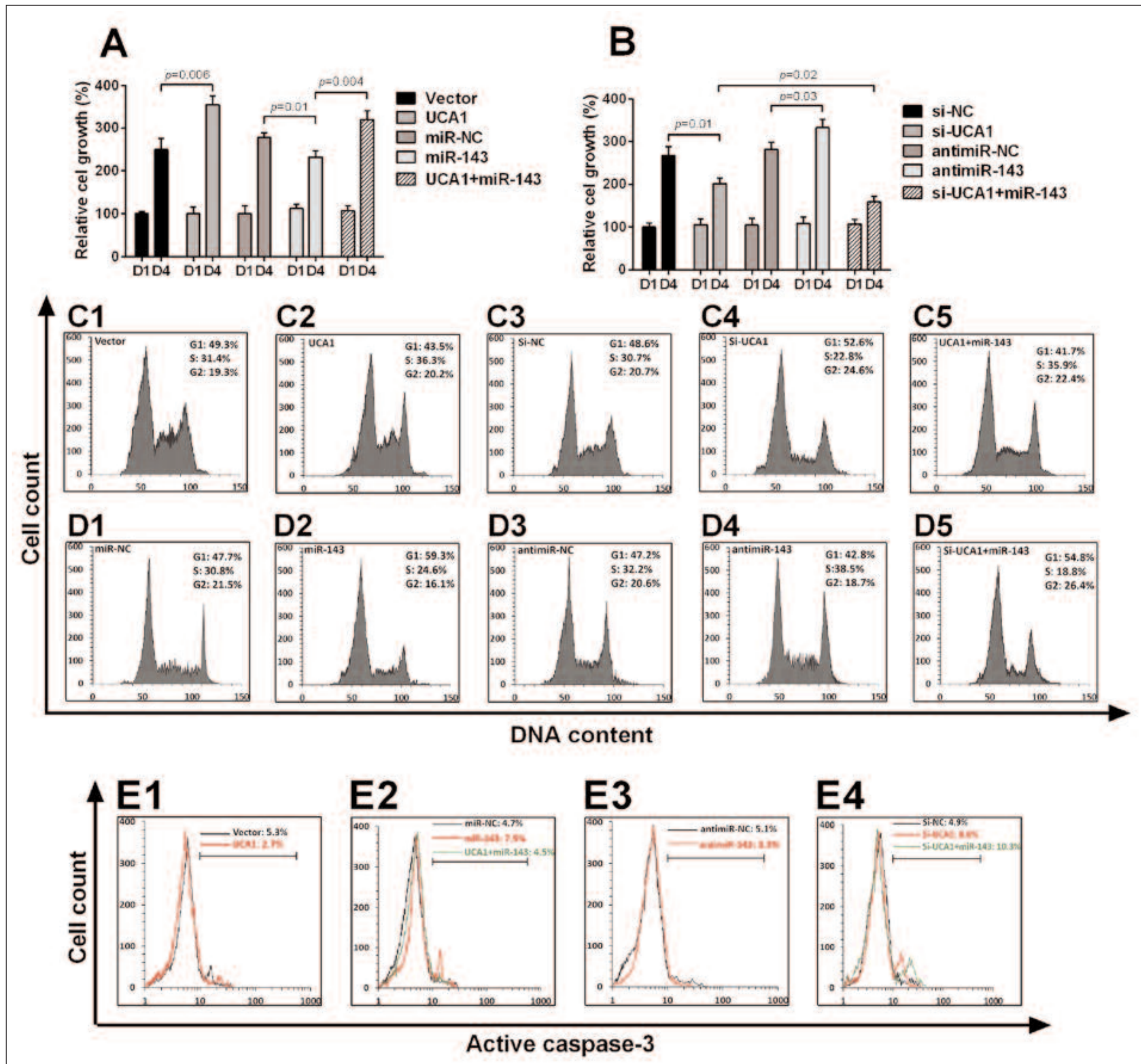

Figure 3. UCA1 modulate breast cancer cell growth and apoptosis at least partially through miR-143. MDA-MB-231 cells were transfected with miR-143 mimics, antimiR-143, si-UCA1 or co-transfected with miR-143 mimics and si-UCA1. Besides, the cells were also infected with UCA1 lentiviral particles. The cells with UCA1 overexpression was further transfected with miR-143 mimics. ***A,*** and ***B,*** MTT assay of cell viability of MDA-MB-231 cells with indicated transfection. ***C1-C5*** and ***D1-D5*** Representative images of flow cytometry analysis of cell cycle phases of MDA-MB-231 cells with indicated transfection. ***E1-E4*** Representative images of flow cytometry analysis of apoptotic MDA-MB-231 cells with indicated transfection.

fection of miR-143 mimics had little influence over cell viability (Figure 3A). Simultaneously knockdown of UCA1 and overexpression of miR-143 has synergy effect in inhibiting cell growth (Figure 3B). Previous study[12] reported that UCA1 can modulate p27 expression, a tumor suppressor controlling cell cycle transition. In this study, we also confirmed that Ectopic expression of UCA1 increased the proportion of breast cancer cells in S phase (vector control *vs.* UCA1: 31.4% *vs.* 36.3%), while decreased cells in G1 population (vector control *vs.* UCA1: 49.3% *vs.* 43.5%) (Figure. 3 C1 and C2). Si-UCA1 showed the opposite effects. Knockdown of endogenous UCA1 decreased the cells in S phase and increased cells in G1 phase (Figure 3 C3 and C4). Ectopic expression of miR-143, had a similar effect as si-UCA1, which decreased the

cells in S phase (miR-NC *vs.* miR-143: 30.8% *vs.* 24.6%) and increased cells G1 phase (miR-NC *vs.* miR-143: 47.7% *vs.* 59.3%) (Figure 3 D1 and D2). In contrast, inhibition of endogenous miR-143 resulted in increased S phase accumulation and decreased G1 population (Figure 3 D3 and D4). However, overexpression of miR-143 could not reverse the cell cycle change due to UCA1 overexpression (Figure 3 C5), but it showed stronger effect when combined with si-UCA1 than either si-UCA1 or miR-143 alone (Figure 3 C4, D2 and D5). We further studied the how the UCA1-miR-143 axis affects cell apoptosis by flow cytometry analysis of apoptotic cells. Results showed that ectopic expression of UCA1 or suppressing endogenous miR-143 decreased apoptosis (Figure 3 E1 and E3). In comparison, knockdown of endogenous UCA1 or overexpression of miR-143 increased the ratio of apoptotic cells (Figure 3 E2 and E4). Overexpression of UCA1 abrogated the effect of miR-143 in inducing cell apoptosis (Figure 3 E2). Simultaneously knockdown of UCA1 and overexpression of miR-143 further enhanced cell apoptosis (Figure 3 E4). These results suggest that the UCA1 can modulate breast cancer cell growth and apoptosis at least partially through miR-143.

Discussion

The pathological development of breast cancer is a complex multistep process, involving a series of dysregulated tumor suppressor genes and oncogenes. One recent study[21] based on microarray found 790 up-regulated and 637 down-regulated lncRNAs with at least 2.3 fold change between breast cancer tissues and the paired adjacent tissues. However, the biological functions of them in tumorigenesis and tumor progression are still poorly understood[22].

lncRNAs may take part in tumor development, recurrence, metastasis, and prognosis through multiple levels, including transcriptional, post-transcriptional and epigenetic regulation. At transcriptional level, for example, Forkhead box C1 (FOXC1) is well known as an oncogene of basal-like breast cancer (BLBC): Lin et al[23] found a novel lncRNA, FOXC1 promoter upstream transcript (FOXCUT) can significantly promote FOXC1 expression, thereby enhancing cancer cell proliferation and migration. ZNF703 expression, a common Luminal B breast cancer oncogene[5]. LncRNA SPRY4-IT1 can target ZNF703 and increase its expression, leading to enhanced proliferation of human breast cancer cells[4]. At post-transcriptional level, the concept of ceRNA was proposed to explain the indirect regulative function of lncRNAs. One recent study[8] found ectopic expression of lncRNA linc-ROR in immortalized human mammary epithelial cells induced EMT of the cells. In addition, increased linc-ROR expression also enhanced breast cancer cell migration and invasion, showing certain level of stem cell properties. Linc-ROR functions as a competing endogenous RNA to miR-205, thereby preventing degradation of miR-205 target genes, including the EMT inducer ZEB2. LncRNA ATB can increase trastuzumab resistance and invasion-metastasis cascade in breast cancer via competitively biding miR-200c, leading to subsequently upregulation of ZEB1 and ZNF-217, which induce EMT[9].

We observed inverse expression between lncRNA UCA1 and miR-143. This triggered our interest to explore whether there is a ceRNA mechanism involved between UCA1 and miR-143. The oncogenic role of UCA1 is widely reported in several types of cancer. For example, UCA1 is upregulated in colorectal cancer, bladder cancer and ovarian cancer, promoting cell proliferation, suppressing apoptosis and promote cell cycle progression[24-26]. In gastric cancer, high UCA1 expression is correlated with worse differentiation, tumor size, invasion depth and TNM stage[27]. In metastatic breast cancer cells, UCA1 is necessary for maintaining tumorigenic activity[28]. One recent study[12] found the 1.4 kb isoform of UCA1 has an oncogenic role in breast cancer by forming a complex with heterogeneous nuclear ribonucleoprotein I (hnRNP I), downregulating p27 expression. However, whether other mechanisms are involved in its regulation over breast cancer is not clear. We found that there are direct interactions between the 1.4 kb isoform of UCA1 and miR-143. UCA1 and miR-143 are in the same RISC complex. Therefore, overexpression of UCA1 might be a mechanism of lowered miR-143 level in breast cancer. The tumor suppressive role of miR-143 in breast cancer was reported in previous studies. miR-143 and miR-145 can simultaneously target 3'TUR of the ERBB3 gene and regulate ERBB3 expression, thereby suppressing the proliferation and invasion of breast cancer cells[16]. In addition, miR-143 can also regulate BCL-2, inhibiting E(2)-induced cell proliferation[29]. Therefore, it is possible that through regulating miR-143, UCA1 can indirectly modu-

late cell proliferation of breast cancer cells. Then we further verified this hypothesis in MDA-MB2-231 cells. Results confirmed that UCA1 can modulate breast cancer cell growth and apoptosis at least partially through miR-143.

Conclusions

This report revealed a novel mechanism of UCA1 in tumorigenesis of breast cancer. UCA1 can directly interact with miR-143, lowering its expression and thus affect its downstream regulation. Therefore, the UCA1-miR-143 axis constitutes a part of the oncogenic role of UCA1 in breast cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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