

LncRNA UCA1 promotes epithelial-mesenchymal transition (EMT) of breast cancer cells via enhancing Wnt/beta-catenin signaling pathway

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Abstract. – **OBJECTIVE:** LncRNA UCA1 can promote invasion of breast cancer cells. However, the underlying mechanism is not quite clear. In this study, we investigated the regulative effect of UCA1 on the invasion capability of breast cancer cells and its association with the Wnt/ β -catenin pathway.

MATERIALS AND METHODS: Human breast cancer cell line MDA-MB-231 cells were transfected for UCA1 knockdown using UCA1 si-RNA. Transwell assay was performed to assess cell invasion capability. Western blot analysis was conducted to investigate the expression of mesenchymal and epithelial markers and the proteins involved in Wnt/ β -catenin signaling pathway. Immunofluorescent staining was further performed to verify the expression of E-cadherin and N-cadherin.

RESULTS: MDA-MB-231 cells have strong invasion capability. Knockdown of endogenous UCA1 significantly reduced the number of invading cells. MDA-MB-231 cells with UCA1 knockdown had significantly increased expression of E-cadherin but decreased expression of N-cadherin, Vimentin and Snail. UCA1 inhibition substantially increased the expression of p-GSK-3 β and GSK-3 β and suppressed the protein expression of β -catenin and transcription of the downstream genes, including cyclin D1 and MMP-7.

CONCLUSIONS: UCA1 can modulate epithelial-mesenchymal transition (EMT) of MDA-MB-231 cells and knockdown of UCA1 impaired the mesenchymal properties. UCA1 upregulation increases invasiveness of breast cancer cells at least partly via activating the Wnt/ β -catenin signaling pathway.

Key Words: Breast cancer, EMT, UCA1, Wnt/ β -catenin.

Introduction

Long non-coding RNAs (lncRNAs) are evolutionarily conserved non-protein-coding RNAs

greater than 200 nucleotides¹. There are emerging evidence showed that lncRNAs are involved in diverse physiological processes including cell differentiation and development and tumorigenesis via transcriptional and epigenetic regulatory mechanisms²⁻⁴.

Urothelial carcinoma-associated 1 (UCA1) is a lncRNA with three exons that encode a 1.4 kb isoform and a 2.2 kb isoform⁵. Some recent studies suggest that the 1.4 kb isoform of UCA1 acts as an oncogene in breast cancer. Briefly, UCA1 can competitively interact with hnRNP I and thereby suppress the p27 protein level, leading to enhanced breast tumor growth⁵. In addition, it can also promote breast cancer cell growth and reduce cell apoptosis via decreasing tumor suppressive miR-143⁶. One recent study observed that activation of UCA1 can promote invasion of breast cancer cells⁷. However, the underlying mechanism is not quite clear.

Wnt signaling pathway is a highly conserved signaling pathway that regulates multiple biological processes, such as cell proliferation, differentiation, and cell fate in both normal development and disease progression⁸. Wnt/ β -catenin pathway plays a critical role in the development and progression of breast cancer^{9,10}. In this signaling pathway, β -catenin functions as a key signaling mediator. Activation of canonical Wnt signaling results in increased level of β -catenin in both cytoplasm and nucleus, which ultimately activates transcription of the downstream target genes, including C-myc, Cyclin D1, matrix metalloprotease-7 (MMP-7) and others¹¹. In breast cancer tissues, β -catenin is usually aberrant activated. Inhibition of the Wnt/ β -catenin pathway can reduce the epithelial-mesenchymal transition

(EMT) and suppress invasion of breast cancer cells^{12,13}.

In this work, we investigated the regulative effect of UCA1 on invasion capability of breast cancer cells and further studied its association with the Wnt/ β -catenin pathway.

Materials and Methods

Cell Culture and Transfection

Human breast cancer cell line MDA-MB-231 cells were obtained from ATCC and were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 μ g/mL streptomycin and 100 U/mL penicillin in a humidified atmosphere with 5% CO₂ at 37°C.

UCA1 siRNA and the scramble negative controls were purchased from Ribobio (Guangzhou, China). MDA-MB-231 cells were transfected with 100 nM UCA1 si-RNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Transwell Assay of Cell Invasion

Transwell assay follows the methods introduced in one previous study¹⁴. In brief, the Transwell insert chamber coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used according to manufacturer's instruction. The invading cells on the bottom side were fixed with 4% polyoxymethylene and stained with 0.1% crystal violet. Cell counting was performed at 100 \times magnification under a microscope.

Western Blot Analysis

48 hours after transfection, MDA-MB-231 cells were lysed using a lysis buffer (Beyotime, Shanghai, China) for protein extraction. The protein samples were separated by 10% SDS-PAGE gel and then transferred to nitrocellulose (NC) membrane for a conventional Western blotting analysis. The primary antibodies used include anti-E-cadherin (#3195, Cell Signaling, Danvers, MA, USA), anti-N-cadherin (#13116, Cell Signaling), anti-Vimentin (#5741, Cell Signaling), anti-Snail (#9585, Cell Signaling), anti-p-GSK-3 β (ab9769, Abcam, Cambridge, UK), anti-GSK-3 β (ab32391, Abcam), anti- β -catenin (ab32572, Abcam), anti-Cyclin D1 (EPR2241, Abcam), anti-MMP-7 (ab4044, Abcam) and anti- β -actin (ab3280, Abcam). The signals were visualized by using the ECL Western blotting

substrate (Promega, Madison, WI, USA) and the gray scale of the protein bands were quantified by using the Image-J software.

Fluorescence Microscopy

MDA-MB-231 cells after transfection were grown on coverslips. Then, the cells were fixed in methanol, permeabilized in 0.1% Triton X-100 and blocked in 1% BSA. The expression of E-cadherin and N-cadherin was detected using primary antibodies against E-cadherin (#3195, Cell Signaling) and N-cadherin (#13116, Cell Signaling) respectively at 4°C overnight. After the incubation, the coverslips were washed and further incubated with secondary Anti-Rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor[®] 488 Conjugate) (#4412, Cell Signaling) and Anti-Rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor[®] 555 Conjugate) (#4413, Cell Signaling) respectively for 1 hour at room temperature in the dark. Nuclei were stained using Prolong[®] Gold Antifade Reagent with DAPI (#8961, Cell Signaling). Immunofluorescent images were obtained using an Eclipse Ti-S inverted phase/fluorescent microscope (Nikon, Tochigi, Japan).

Statistical Analysis

Data were presented in the form of means \pm standard deviation (SD) based at least three repeats of three independent studies. One-way ANOVA was performed to compare means of multiple group experiments. Comparison between groups was performed using the unpaired *t*-test. A two-sided *p*-value of <0.05 was considered statistically significant.

Results

UCA1 Modulates Invasion of MDA-MB-231 Cells

Previous studies reported that UCA1 is an oncogenic lncRNA in breast cancer and its upregulation is associated with enhanced invasion of breast cancer cells⁵⁻⁷. However, the exact mechanism is largely unknown. In this study, we firstly investigated the role of UCA1 in the invasion of MDA-MB-231 cells. Transwell assay showed that MDA-MB-231 cells have strong invasion capability (Figure 1A). However, knockdown of endogenous UCA1 significantly reduced the number of invading cells, suggesting weakened tumor cell invasiveness (Figure 1A-B).

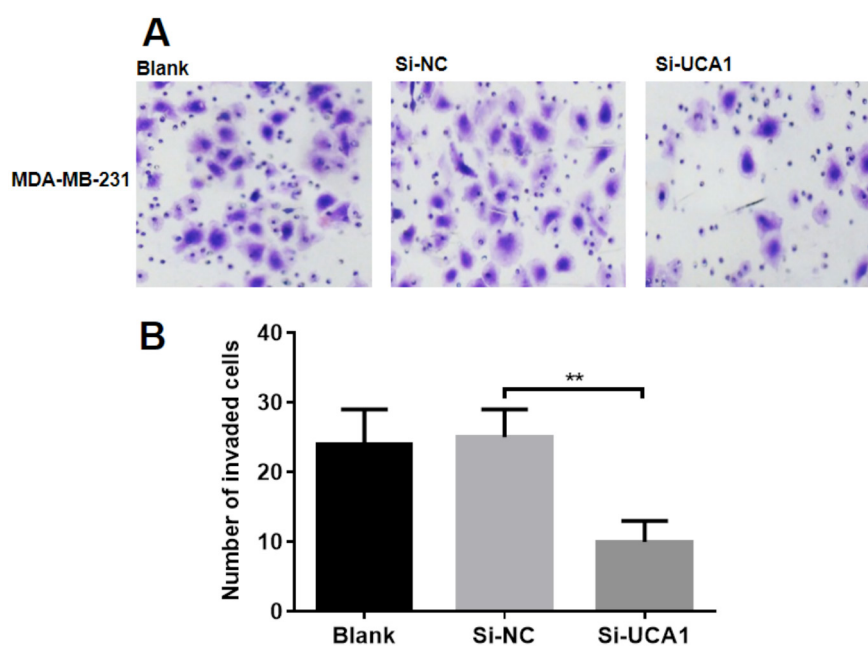


Figure 1. UCA1 modulates invasion of MDA-MB-231 cells. **A**, Representative images of invading MDA-MB-231 cells with or without UCA1 inhibition in transwell invasion assay. **B**, Quantification of invaded cells showed in figure A. ** $p < 0.01$.

Knockdown of UCA1 impaired Mesenchymal Properties of MDA-MB-231 Cells

EMT is an important mechanism of enhanced migration and invasion of cancer cells. Therefore, we further investigated whether UCA1 can modulate EMT of MDA-MB-231 cells. Western blot analysis showed that MDA-MB-231 cells with UCA1 knockdown had significantly increased expression of E-cadherin, a marker of epithelial cell (Figure 2A-B). Meanwhile, UCA1 knockdown also resulted in decreased expression of N-cadherin, Vimentin and Snail, three mesenchymal markers (Figure 2A-B). To further verify these changes, we performed immunofluorescence study to examine the expression of N-cadherin and E-cadherin. The results confirmed that UCA1 knockdown led to increased expression of E-cadherin and reduced expression of N-cadherin in the cells (Figure 2C). These results suggest that UCA1 can modulate EMT of MDA-MB-231 cells and knockdown of UCA1 impaired the mesenchymal properties.

UCA1 Modulates Wnt/beta-Catenin Signaling pathway in MDA-MB-231 Cells

Wnt/beta-catenin signaling pathway is a well-recognized pathway involved in EMT and metastasis in breast cancer^{12,15,16}. Therefore, we decided to investigate the regulative effect of UCA1

on Wnt/beta-catenin signaling. MDA-MB-231 cells transfected with si-UCA1 had substantially increased expression of p-GSK-3 β and GSK-3 β (Figure 3A and C), the negative regulators of the Wnt signaling pathway¹⁷. At the same time, UCA1 knockdown suppressed the protein expression levels of β -catenin and also led to decreased transcription of the downstream genes of β -catenin, including cyclin D1 and MMP-7 (Figure 3B and D). These data indicate that UCA1 silencing inhibits Wnt/ β -catenin activation.

Discussion

The association between UCA1 upregulation and enhanced metastatic potentials of cancer cells was observed in multiple types of cancer, including tongue squamous cell carcinomas¹⁸, ovarian cancer¹⁹, bladder cancer²⁰, melanoma²¹ and breast cancer⁷. In breast cancer, one recent study observed that macrophage infiltration can activate UCA1 and promote invasion of the cancer cells⁷. In this study, by performing transwell assay, we also confirmed that UCA1 can modulate invasiveness of breast cancer cells.

Due to the complex regulative mechanisms of lncRNAs, how aberrant UCA1 expression contributes to enhanced cancer cell migration and

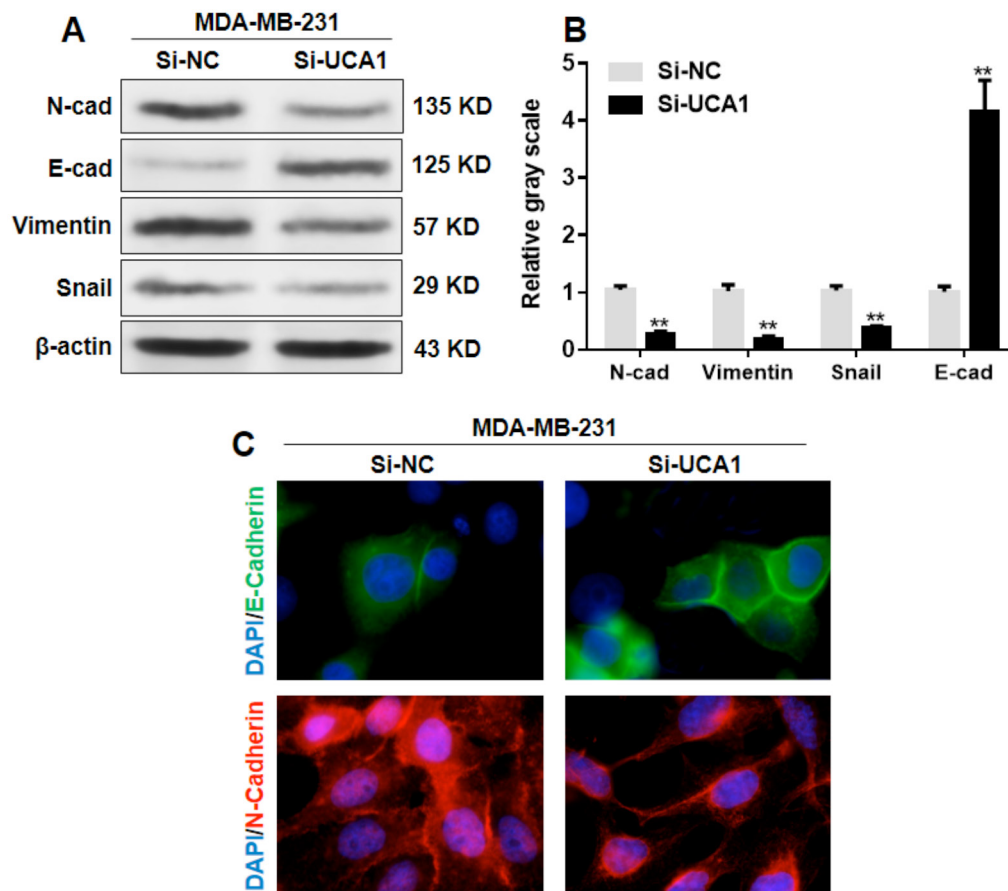


Figure 2. Knockdown of UCA1 impaired mesenchymal properties of MDA-MB-231 cells. *A*, Western blot analysis of N-cadherin (N-cad), E-cadherin (E-cad), Vimentin, Snail and β-actin in MDA-MB-231 cells 48 hours after transfection with UCA1 siRNA (100 nM) or the negative control. *B*, Measurement of the relative gray scale of N-cad, E-cad, Vimentin and Snail vs. β-actin showed in figure A. *C*, Representative images of immunofluorescence staining of E-cadherin and N-cadherin in MDA-MB-231 cells with or without UCA1 inhibition. Red: N-cadherin, Green: E-cadherin, Blue: DAPI. ** $p < 0.01$.

metastasis are not fully understood. In bladder cancer, there is a reciprocal negative regulation of UCA1 and hsa-miR-145. UCA1 represses hsa-miR-145 expression to upregulate ZEB1/2, thereby enhancing bladder cancer cell migration and invasion²⁰. In addition, through the miR-145/ZEB1/2/FSCN1 pathway, UCA1 can also modulate EMT of bladder cancer cells²⁰. In non-small cell lung cancer cells, the association between UCA1 upregulation and enhanced EMT was also observed²². In fact, EMT is an important mechanism contributing to increasing cell invasion and metastatic potential of cancer cells. Therefore, we decided to further investigate the regulative effect of UCA1 on EMT in breast cancer cells. Both Western blot and immunofluorescence results confirmed that UCA1 knockdown impaired mesenchymal properties but induced the expression of the epithelial marker in MDA-MB-231

cells. Then, we decided to investigate further the mechanism involved in the regulative effect of UCA1 on EMT.

The Wnt/β-catenin signaling pathway is usually aberrantly activated in breast cancer and contribute to tumor initiation and progression^{9,10}. Activation of this signaling pathway leads to nuclear translocation of β-catenin²³. Subsequently, β-catenin binds to the Tcf and Lef transcription factors in the nucleus, leading to transcription of its downstream genes, including C-myc, Cyclin D1, MMP-7 and others^{11,23}. In fact, this signaling pathway is involved in regulation of EMT in breast cancer. Previous reports^{12,13,15} showed that inhibition of the Wnt/β-catenin pathway can reduce EMT and suppress invasion of breast cancer cells. Whether UCA1 has a regulative effect on this signaling pathway is still obscure. Only one

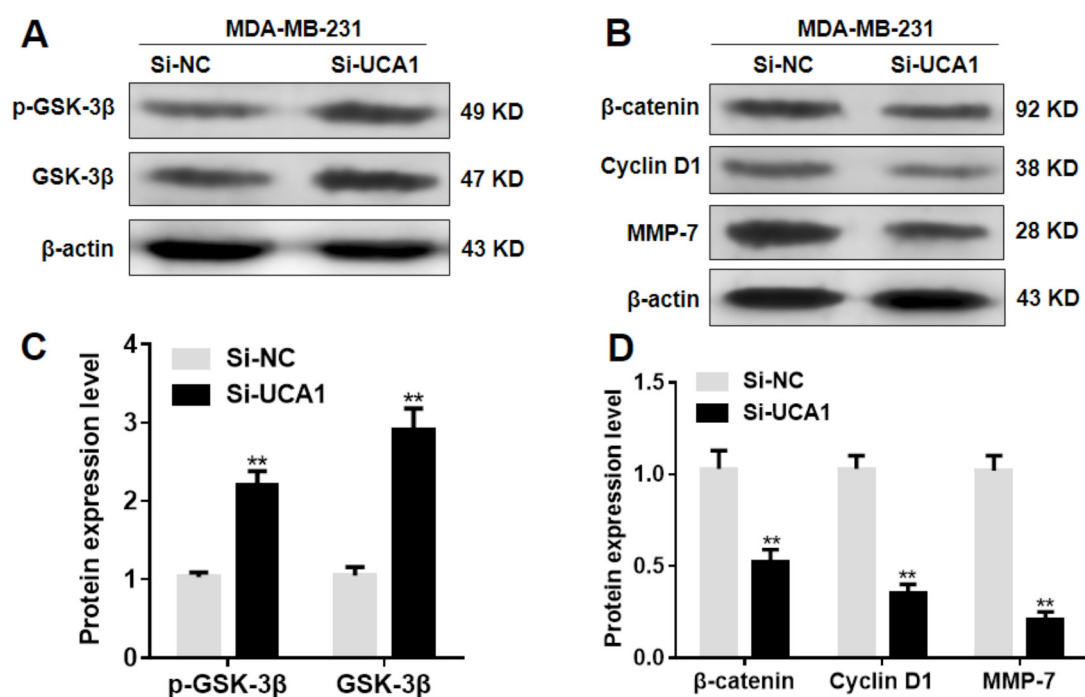


Figure 3. UCA1 modulates Wnt/beta-catenin signaling pathway in MDA-MB-231 cells. *A-B*, Western blot analysis of p-GSK-3β, GSK-3β (A) and β-catenin, Cyclin D1, and MMP-7 (B) in MDA-MB-231 cells 48 hours after transfection with UCA1 siRNA (100 nM) or the negative control. *C-D*, Measurement of the relative protein expression of p-GSK-3β, GSK-3β (C) and β-catenin, Cyclin D1, and MMP-7 (D) vs. β-actin showed in figure A-B. ***p* < 0.01.

study reported that UCA1 can increase the expression of Wnt6, thereby activating Wnt signaling and leading to chemoresistance in bladder cancer²⁴. In this study, we further investigated how UCA1 modulates the Wnt/β-catenin signaling pathway in breast cancer cells. Western blot analysis showed that si-UCA1 substantially increased expression of p-GSK-3β and GSK-3β, the negative regulators of the Wnt signaling pathway which initiate phosphorylation of β-catenin and subsequently β-TrCP1-mediated degradation¹⁷. Besides, UCA1 knockdown also suppressed the protein expression levels of β-catenin and resulted in decreased transcription of the downstream genes of β-catenin, including cyclin D1 and MMP-7. Based on these findings, we infer that UCA1 upregulation increases invasion of breast cancer cells at least partly via activating the Wnt/β-catenin signaling pathway.

Conclusions

UCA1 can modulate EMT of MDA-MB-231 cells and knockdown of UCA1 impaired the mes-

enchymal properties. UCA1 upregulation increases invasion of breast cancer cells at least partly via activating of the Wnt/β-catenin signaling pathway.

Conflicts of interest

The authors declare no conflicts of interest.

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