ANTICANCER RESEARCH *38*: 3427-3434 (2018)
doi:10.21873/anticanres.12611

Silencing *lnc-ASAH2B-2* Inhibits Breast Cancer Cell Growth *via* the mTOR Pathway

JINGJING LI[1,2*], JING ZHANG[1,3*], LIANG JIN[1,2*], HERAN DENG[1,2] and JIANNAN WU[1,2]

[1]Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, [2]Breast Tumor Center, and [3]Anesthesiology Department, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, P.R. China

Abstract. *Background/Aim: Persistent activation of the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (mTOR) pathway is an important mechanism in resistance of breast cancer to endocrine therapy. Although everolimus has potent inhibitory effects on the mTOR pathway, it has demonstrated only modest clinical activity as a single agent. Whether long noncoding (lnc) RNA is involved in everolimus resistance is unknown. Materials and Methods: Cell viability, colony formation and cell proliferation experiments were used to measure the effects of long noncoding RNA N-acylsphingosine amidohydrolase 2B-2 (lnc-ASAH2B-2) knockdown in BT474 and MCF7 breast cancer cells. Results: lnc-ASAH2B-2 was up-regulated by everolimus in cells with and without serum, and reduction of lnc-ASAH2B-2 expression was able to inhibit proliferation of BT474 and MCF7 cells. Conclusion: lnc-ASAH2B-2 was up-regulated after everolimus exposure and efficiently regulated breast cancer cell growth by activating the mTOR pathway, which may reduce the effect of everolimus, providing evidence that lnc-ASAH2B-2 might be a new therapeutic target for breast cancer.*

Breast cancer is one of the three most prevalent malignancies worldwide, second only to lung and colon cancer (1). Nearly 70% of breast cancer cases are of the luminal subtype, hormone receptor positive and human epidermal growth factor receptor 2 (HER2)-negative, and it has higher incidence among postmenopausal women (2). This subtype of breast cancer usually has a positive response to hormonal treatments such as tamoxifen, fulvestrant and exemestane, but some tumours develop *de novo* or acquired resistance to endocrine therapy (3). Persistent activation of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway is an important mechanisms for resistance to endocrine therapy (4).

Substantial evidence has demonstrated that the mTOR pathway plays a pivotal role in tumourigenesis, as it controls many cellular processes such as protein synthesis, cellular metabolism and autophagy (5). As an allosteric inhibitor of mTOR, everolimus potently inhibits tumour growth because it can impair cancer proliferation and metabolism (6). In clinical trials, the combination of everolimus and exemestane has been shown to restore hormone sensitivity in patients with advanced hormone receptor-positive breast cancer resistant to hormone therapies, while everolimus as a single agent has demonstrated only modest clinical activity (7, 8). One of the reasons for its limited clinical success is that everolimus was found to relieve negative feedback loops thereby activating PI3K signalling (6), but more mechanisms need to be further explored.

Currently, long noncoding RNAs (lncRNAs) have been validated as key regulators in differentiation, development, and disease. lncRNAs are a family of transcripts that are longer than 200 nucleotides and contain no open reading frame, and they are less abundant and more variable among tissues than mRNA expression (9, 10). Recent studies have shown that lncRNAs can act as signals, decoys, guides and scaffolds to modulate the transcriptional or post-transcriptional regulation of gene expression in multiple cancer types, making them good prognostic biomarkers and therapeutic targets (11-13). For example, HOX transcript antisense RNA functions as a scaffold by interacting with both polycomb repressive complex 2 and lysine-specific histone demethylase 1 to modulate chromatin states (14). Nuclear factor κB-interacting lncRNA suppresses breast cancer metastasis by regulating NFκB activation (15).

*These Authors contributed equally to this study.

Correspondence to: Jiannan Wu, Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Breast Tumor Center, Sun Yat-Sen Memorial Hospital, Sun-Yat-Sen University, Guangzhou 510120, P.R. China. E-mail: wujiann3@mail.sysu.edu.cn

Key Words: Breast cancer, everolimus, long noncoding RNA, lnc-ASAH2B-2, therapeutic target.

Prostate cancer-associated non-coding RNA 1 and prostate-specific transcript 1 bind to androgen receptor and enhance both gene activation programmes and prostate cancer cell growth (16).

Therefore, whether IncRNA is involved in the regulation of everolimus needs to be further investigated. In this study, we investigated whether one lncRNA is involved in regulation of breast cancer cell growth *via* the mTOR pathway.

Materials and Methods

Cell lines and inhibitor. BT474 (ER-positive, *HER2* amplification) and MCF7 (ER-positive, no amplification of *HER2*) breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium (Gibco, Carlsbad, CA, USA) containing 10% foetal bovine serum (Gibco) at 37˚C in a 5% CO_2 incubator. Everolimus (NVP-RAD001, A8169) was purchased from APExBIO (Houston, TX, USA) and dissolved in dimethyl sulfoxide (Sigma-Aldrich, Shanghai, China).

HTA 2.0 transcriptome microarray assay of lncRNAs. After 24 h of 10 nM everolimus treatment, total RNA of BT474 and MCF7 cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and 5 μg samples of total RNA were subjected to Affymetrix GeneChip Array (Human Transcriptome 2.0; Affymetrix, Santa Clara, CA, USA). LncRNAs that differed in expression by more than 1.5-fold under everolimus treatments *versus* DMSO for both cell types were selected. Heat maps representing differentially regulated lncRNAs were generated using HemI (Heatmap Illustrator, version 1.0, CUCKOO Workgroup, Hubei, PR China) (17).

Small interfering (si) RNA transfection. For this purpose, 2×10^5 BT474 and MCF7 cells were plated in 6-well plates for 24 h before transient transfection with siRNA. Cells were transfected using Lipo3000 (Invitrogen) with 50 pmol of oligonucleotides in 5 μl reactions according to the manufacturer's protocols. The cells were harvested after 72 h for quantitative real-time polymerase chain reaction (qRT-PCR) and western blot assays. The nucleotide sequences of the siRNAs designed by GenePharma (Hangzhou, China) were: lnc-ASAH2B-2 si1: GCAGUGAGCAAGAGAACUATT, lnc-ASAH2B-2 si2: CCGUAUCCAUAUUCUACAATT

Real-time PCR and qRT-PCR. Cells were treated with everlimus. Total RNA from BT474 and MCF7 cells was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed into first-strand cDNA using M-MLV Transcriptase Kit (Invitrogen) according to the manufacturer's protocols. qRT-PCR was performed using a LightCycler® 480 II system (Roche, Basel, Switzerland) with SYBR® Green Real-time PCR Master Mix (Toyobo, Osaka, Japan). The primers were: lncRNA N-acylsphingosine amidohydrolase 2B-2 (lnc-ASAH2B-2) forward: GGATGGCGATGGTCAGGAAA, reverse: TTGGAGAGAGTTGCTTGGTG; β-actin (*ACTB*) forward: TGA AGTGTGACGTGGACAT, reverse: GGAGGAGCAATGATCTTG AT. *ACTB* served as an internal control.

Cell viability assay. After siRNA transfection for 24 h, 1,000 cells per well were reseeded into 96-well plates in 100 μl of complete culture medium and cultured for another 24, 48 or 72 h. Then, 100 μl of CellTiter-Glo reagent (Promega, Madison, WI, USA) was added to each well, and the luminescence values were recorded on a Mithras^2 LB 943 (Berthold Technologies, Bad Wildbad, Germany). Luminescence values were used to compare the cell viability relative to the control cells. All experiments were performed in triplicate.

Cell proliferation assay. Cells were transfected with siRNA, then trypsinized at 72 h and counted on a Coulter counter. All experiments were performed in triplicate.

Colony-formation assay. After siRNA transfection for 24 h, single-cell suspensions were plated in 6-well plates at a density of 1,000 cells per well and maintained in complete culture medium, which was replaced every 3 days. Two weeks later, cell colonies were stained with 0.5% crystal violet staining solution for 10 minutes. Plates were then scanned using an HP printer to image the results. All experiments were performed in triplicate.

Western blot. BT474 and MCF7 cells were lysed on ice with RIPA buffer containing protease inhibitor cocktail (Pierce, Rockford, IL, USA), and the lysates were cleared by centrifugation for 20 min at 13,000 rpm. The protein concentration in each sample was quantified with BCA Kit (Pierce, Rockford, IL, USA). Total protein (25-50 μg) was loaded onto 8% or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membranes. The blots were blocked in 5% non-fat milk for 1 h and then probed with primary antibodies overnight at 4˚C. Western blot determinations were carried out using primary antibodies purchased from Cell Signaling Technology (Danvers, MA, USA) to the following: mTOR (1:1,000), phospho-mTOR (Ser2448) (1:1,000), p70S6 (1:1,000), phospho-p70S6 (Thr389) (1:1,000), phospho-p70S6 (Ser371) (1:1,000), 4EBP1 (1:1,000), phospho-4EBP1 (Thr37/46) (1:1,000) and β-actin (1:1,000). The immunoblotted proteins were visualized using Dura Chemiluminescence Substrate (Invitrogen) according to the manufacturer's instructions.

Statistical analysis. All statistical analyses were performed using SPSS standard version 16.0 software (SPSS, Chicago, IL, USA). The results are presented as the mean and SD from at least three independent experiments. Comparisons between groups were evaluated by Student's *t*-test. Differences with *p*-values of less than 0.05 were considered statistically significant.

Results

lnc-ASAH2B-2 was up-regulated by everolimus in BT474 and MCF7 cells. As everolimus is widely used to inhibit the activation of the mTOR pathway in clinical treatment, we used it to investigate whether mTOR signalling was inhibited in the BT474 and MCF7 cell lines *via* detecting the phosphorylation of the mTOR substrates p70S6 at T389/S371 and 4EBP1 at T37/46. After everolimus treatment for 24 h, western blot assays showed that the phosphorylation at T389 of p70S6 was dramatically reduced, while the phosphorylation at T37/46 of 4E-BP1 was potently inhibited in a dose-dependent manner.

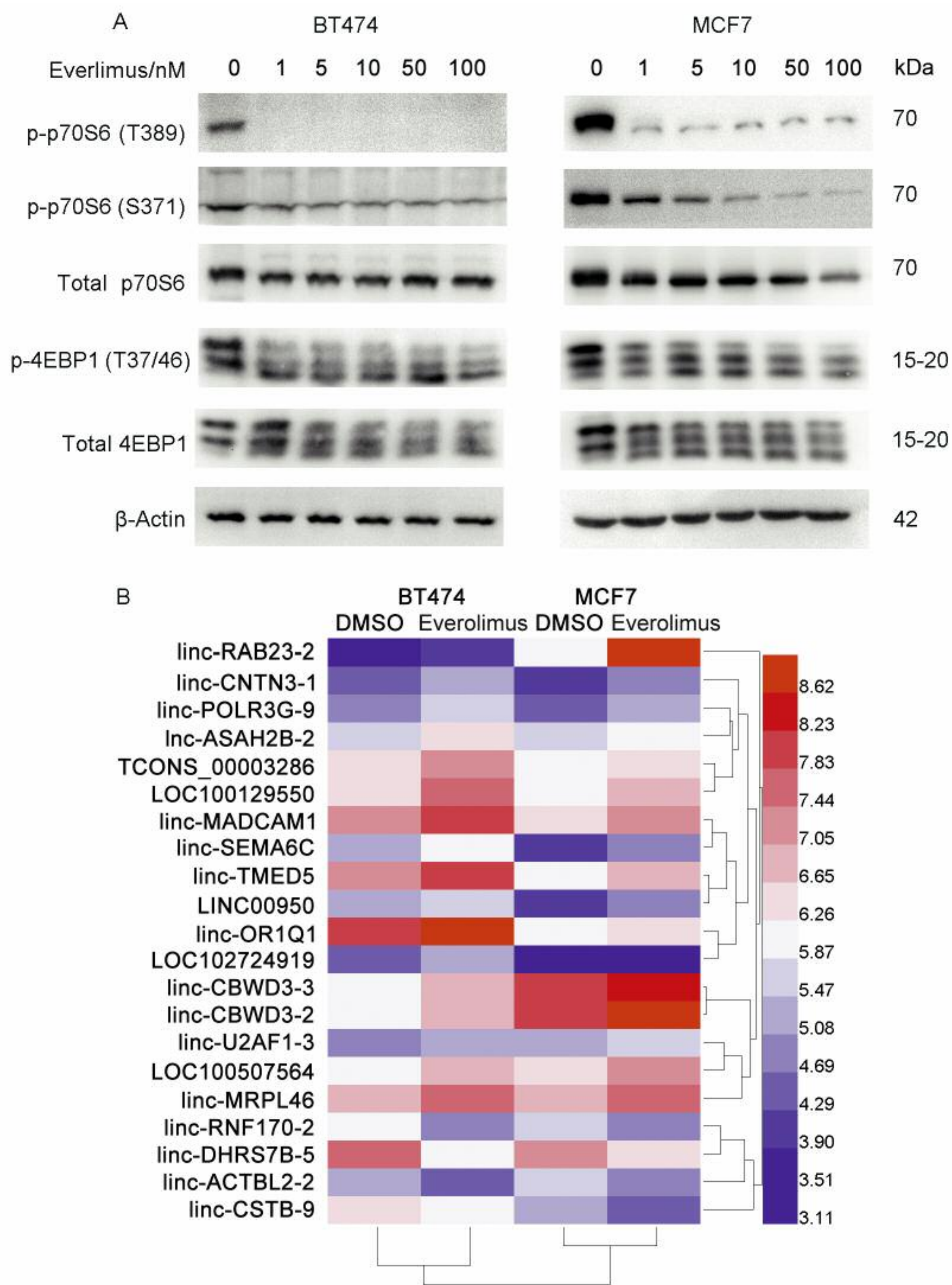

Figure 1. *A: Western blots showed mammalian target of rapamycin (mTOR) substrates were influenced by everolimus in a dose-dependent manner in BT474 and MCF7 cells. Representative images of three independent experiments. B: Heatmap showing the expression of 21 long non-coding RNAs was altered more than 1.5-fold in BT474 and MCF7 cells by treatment with everolimus.*

However, the total protein levels of p70S6 and 4E-BP1 showed little difference (Figure 1A). Therefore, we chose 10 nM everolimus for use in our subsequent experiments, as it efficiently inhibited the mTOR pathway.

To investigate the role of lncRNAs in the mTOR pathway, lncRNA expression profiles were determined using the Human Transcriptome 2.0 microarray after treating BT474 and MCF7 cells with everolimus. The

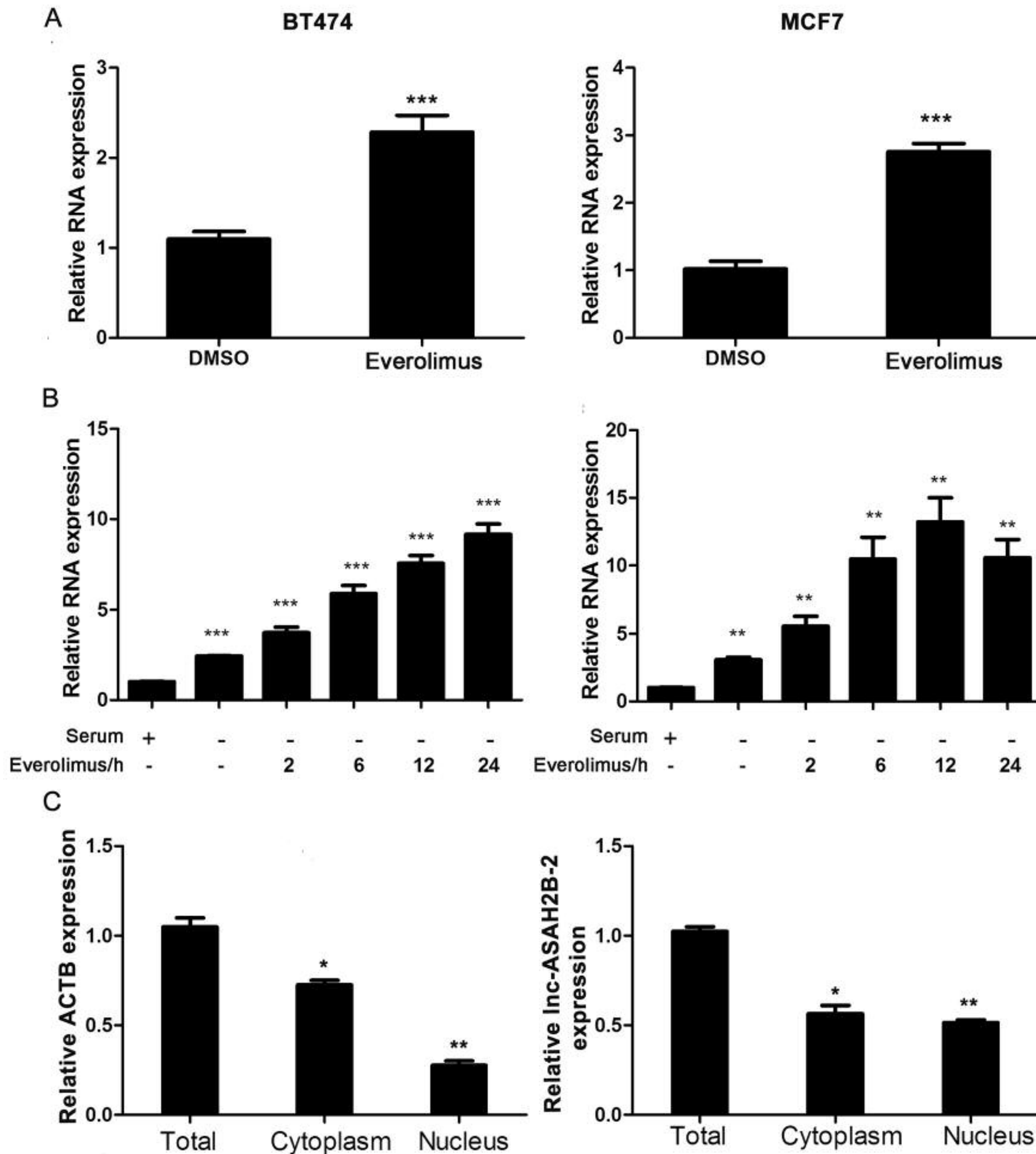

Figure 2. *Long noncoding RNA N-acylsphingosine amidohydrolase 2B-2 (lnc-ASAH2B-2) was up-regulated after everolimus treatment in BT474 and MCF7 cells as shown by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). A: lnc-ASAH2B-2 was increased by 10 nM everolimus treatment in both BT474 and MCF7 cells. B: Lnc-ASAH2B-2 was up-regulated by everolimus under serum starvation in a time-dependent manner. C: β-actin (ACTB) and lnc-ASAH2B-2 were located in both nucleus and cytoplasm of MCF7. Data are the mean±SD of three replicates. Significantly different from the control at *p<0.05, **p<0.01, and ***p<0.001.*

microarray data revealed that 20 lncRNAs were up-regulated and one lncRNA was down-regulated by more than 1.5-fold compared to the control group in both BT474 and MCF7 cells (Figure 1B). Among these lncRNAs, we found *lnc-ASAH2B-2*, which is a IncRNA located at chromosome 10:52386924-52407050, according to the University of California Santa Cruz Genome Browser (http://genome.ucsc.edu), and the probe set ID of *lnc-ASAH2B-2* is TC10002070.hg.1. The qRT-PCR results demonstrated that the expression of *lnc-ASAH2B-2* was increased in both BT474 and MCF7 cells more than two-fold by everolimus treatment (Figure 2A).

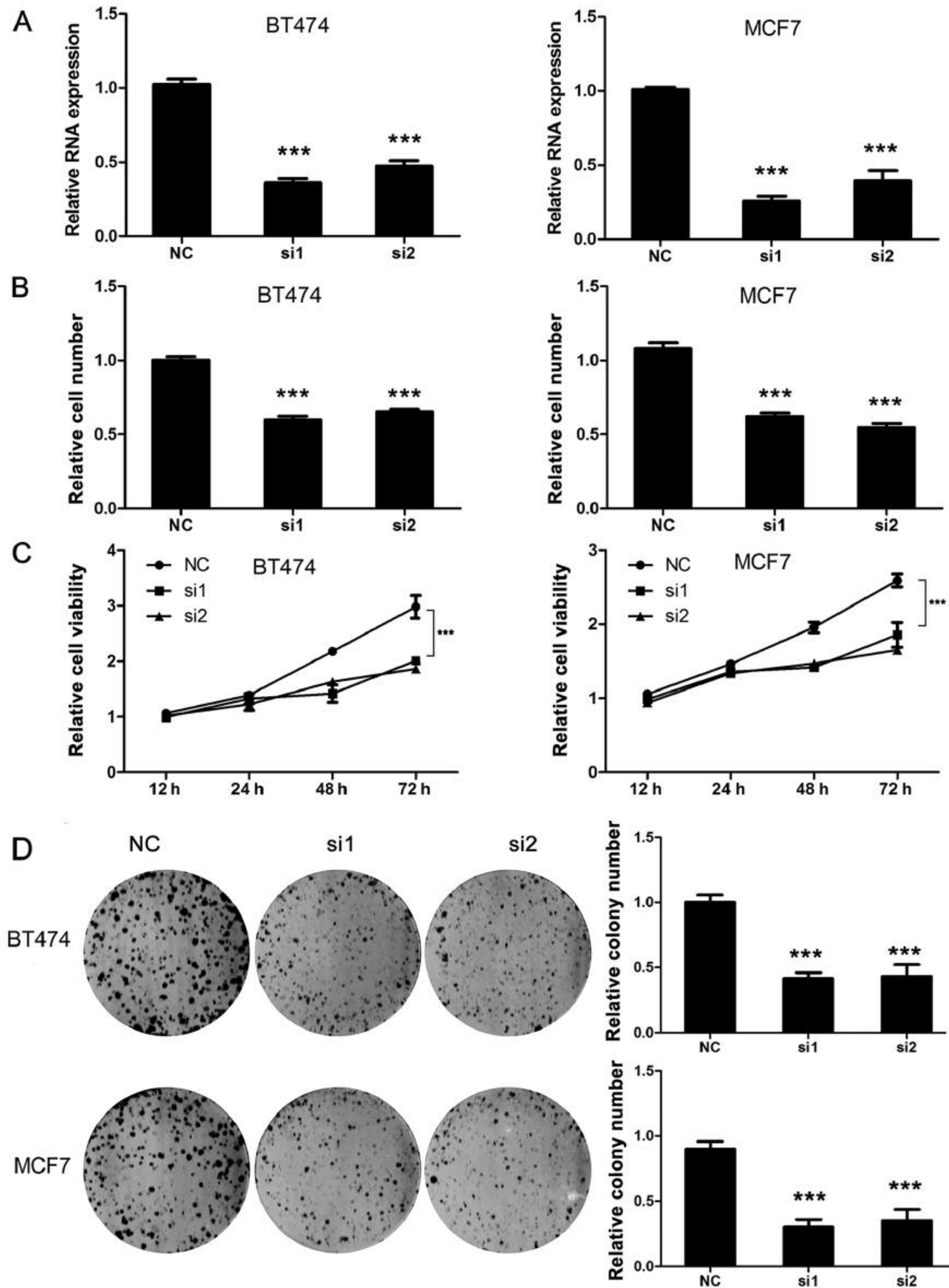

Figure 3. *Knock-down of long noncoding RNA N-acylsphingosine amidohydrolase 2B-2 (lnc-ASAH2B-2) with small interfering (si) RNA inhibited breast cancer cell proliferation. A: Relative expression of lnc-ASAH2B-2 in BT474 and MCF7 cells was significantly reduced by siRNA. B: Cell count assays were performed to determine the proliferation of BT474 and MCF7 cells 72 h after siRNA transfection. C: Cell titer glo assay was performed to determine the proliferation of BT474 and MCF7 cells 72 h after siRNA transfection. D: Colony-formation assays were performed to determine the proliferation of BT474 and MCF7 cells 7 days after siRNA transfection. Data are the mean±SD of three replicates. Significantly different from the control at ***p<0.001.*

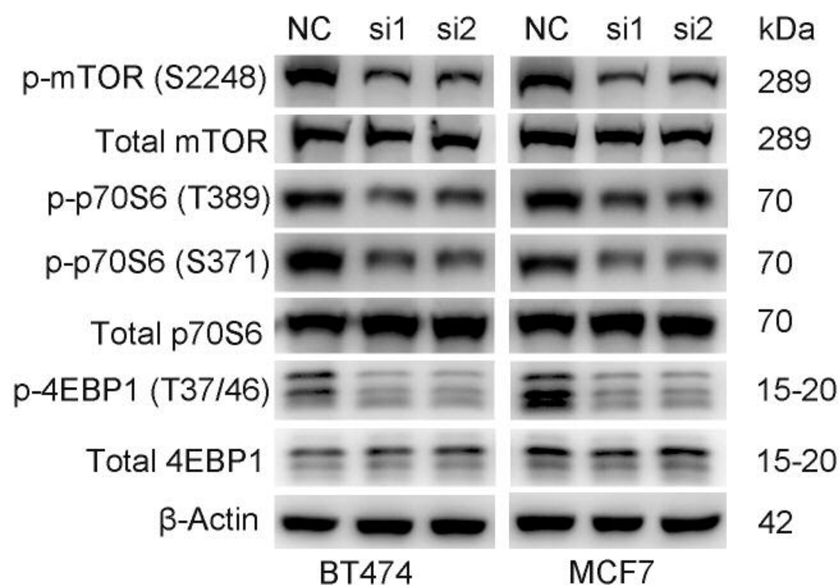

Figure 4. *Long noncoding RNA N-acylsphingosine amidohydrolase 2B-2 (lnc-ASAH2B-2) knock-down efficiently inhibited the expression of mammalian target of rapamycin (mTOR) pathway proteins. Western blots showed siRNA-mediated knock-down of lnc-ASAH2B-2 down-regulated mTOR pathway proteins in BT474 and MCF7 cells. Representative images of three independent experiments.*

lnc-ASAH2B-2 was up-regulated by everolimus under serum starvation. Furthermore, we examined whether *lnc-ASAH2B-2* was regulated under different serum conditions. BT474 and MCF7 cells were treated with 10 nM everolimus for 0, 2, 6, 12, 24 h under serum-starved culture condition. The time-course experiments revealed that *lnc-ASAH2B-2* expression was gradually stimulated under serum-starvation treatment, and expression of *lnc-ASAH2B-2* peaked at 24 h in BT474 and at 12 h in MCF7 cells (Figure 2B). Nuclear/cytoplasmic fractionation confirmed that *ACTB*, used as a positive control, was mostly located in the cytoplasm, while *lnc-ASAH2B-2* was located in both the nucleus and the cytoplasm (Figure 2C), which suggests that it may perform biological functions in the cytoplasm in addition to affecting mTOR.

lnc-ASAH2B-2 functioned as an oncogene in regulating breast cancer cell growth. To verify its biological functions, we used two siRNAs, *lnc-ASAH2B-2* si1 and *lnc-ASAH2B-2* si2, to knock-down *lnc-ASAH2B-2*. qRT-PCR showed that the siRNA targeting of *lnc-ASAH2B-2* reduced its expression by 50% (Figure 3A). Cell counts and cell viability assays were performed to determine the impact of reduced *lnc-ASAH2B-2* expression. The results revealed that the knockdown of *lnc-ASAH2B-2* significantly reduced cell numbers and cell viability of BT474 and MCF7 cells compared to the negative control groups at 72 h (Figure 3B and C). The same phenomenon was also observed in the cell colony-formation assays: the number of colonies formed by BT474 and MCF7 cells transfected with *lnc-ASAH2B-2* si1 and *lnc-ASAH2B-2* si2 were clearly lower than in the negative control groups (Figure 3D). These results suggest that lnc-ASAH2B-2 may function as an oncogene to promote breast cancer cell growth.

lnc-ASAH2B-2 knock-down efficiently inhibited the mTOR pathway. Since *lnc-ASAH2B-2* regulates breast cancer cell growth, we wanted to understand whether it has any effect on the activation state of the mTOR pathway. Therefore, we used a western blot assay to detect mTOR substrates within cells with down-regulation of *lnc-ASAH2B-2*. Figure 4 shows that total mTOR, p70S6 and 4E-BP1 were not apparently altered, but the phosphorylation of mTOR at S2248 and p70S6 at T389/S371 were partially suppressed by *lnc-ASAH2B-2* knockdown, and the phosphorylation at T37/46 of 4E-BP1 was efficiently modulated. These results suggested that *lnc-ASAH2B-2* seems to regulate the mTOR pathway.

Discussion

Recently, a number of lncRNAs have been discovered to be attractive biomarkers for tissue-specific expression and to be potential therapeutic targets in cancer because of their contribution to a wide range of cellular biological processes, such as cell proliferation, metastasis, and survival (18). Other researchers have reported that the lncRNA colorectal neoplasia differentially expressed (CRNDE) promotes glioma cell growth and invasion *via* mTOR signalling (19),

and lncRNA urothelial cancer associated 1 (UCA1) interacts with polypyrimidine tract binding protein 1 to suppress p27 to promote breast cancer cell growth (20).

Since mTOR signalling has been verified to play a significant role in cell proliferation and is aberrantly regulated in breast cancer, the use of mTOR inhibitor everolimus has already become a therapeutic strategy (21). In our study, 10 nM everolimus was found to efficiently suppress mTOR substrates, including p70S6 and 4E-BP1.

In the search for mediators of the mTOR pathway after everolimus exposure, we identified *lnc-ASAH2B-2* as a lncRNA whose expression increased after everolimus exposure in both BT474 and MCF7 cells and to our knowledge this has never previously been reported. Furthermore, we focused on the biological function of *lnc-ASAH2B-2* in breast cancer. Our observations showed that loss of function of *lnc-ASAH2B-2* reduced cell proliferation in both colony-formation and cell-viability assays, suggesting that *lnc-ASAH2B-2* knock-down can suppress the development of breast cancer. To determine the regulation of *lnc-ASAH2B-2* by its downstream molecules, we investigated its influence on the mTOR pathway. The western blot assay showed that the siRNA-mediated silencing of *lnc-ASAH2B-2* significantly reduced the phosphorylation of the mTOR substrates p70S6 and 4EBP-1.

We found that everolimus inhibited the mTOR pathway *via* inhibition of the phosphorylation of mTOR substrates, while *lnc-ASAH2B-2* was up-regulated by everolimus. Interestingly, *lnc-ASAH2B-2* can in turn up-regulate the phosphorylation of mTOR substrates, reducing the inhibition of the mTOR pathway by everolimus. These data establish that *lnc-ASAH2B-2* may participate in a negative feedback loop in response to everolimus exposure.

In several clinical trials, everolimus has achieved only modest effects as a single agent, but it improved patient outcome in HR-positive advanced breast cancer in combination with exemestane (7, 8, 22).The reasons for its limited clinical success have not been fully validated but are likely related to its failure to completely inhibit substrates such as p-4E-BP1 (T37/46) (23) and to its activation of several feedback loops involved in cell survival responses, such as receptor tyrosine kinase-PI3K signalling (24). As everolimus has limited influence on the phosphorylation of 4EBP-1, whereas we show reduction of *lnc-ASAH2B-2* expression potently down-regulated p-4E-BP1 (T37/46), the combination of everolimus and *lnc-ASAH2B-2* knockdown may be worth further investigation. Moreover, we also found that the expression of *lnc-ASAH2B-2* was up-regulated much more strongly by everolimus in cells cultured without serum than with serum, suggesting that it may participate in the regulation of other cellular growth factors in breast cancer. Therefore, the interaction between *lnc-ASAH2B-2* and other growth factors will also be addressed in our upcoming research.

Collectively, with a decrease in *lnc-ASAH2B-2*, the mTOR signalling pathway was suppressed *in vitro*. This demonstrates that *lnc-ASAH2B-2* may promote tumourigenesis *via* regulating the mTOR signalling pathway, which may reduce the effect of everolimus. Therefore, it may play an important role in breast cancer cell growth and resistance to everolimus.

In conclusion, we identified *lnc-ASAH2B-2* as a lncRNA that regulates cell growth *via* the mTOR pathway in breast cancer cells.

Acknowledgements

This work was supported by grants from the Natural Science Foundation of China (81702615), from the Science and Technology Programme of Guangzhou (20170402009), from the Key Laboratory of Malignant Tumour Molecular Mechanism and Translational Medicine of the Guangzhou Bureau of Science and Information Technology ((2013)163), from the Key Laboratory of Malignant Tumour Gene Regulation and Target Therapy of Guangdong Higher Education Institutes (KLB09001), and from the Guangdong Science and Technology Department (2015B050501004).

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Received April 1, 2018
Revised May 3, 2018
Accepted May 9, 2018