Breast cancer is of high incidence and is the leading cause of cancer death among females, with an estimated 1.7 million cases and 521,900 deaths worldwide in 2012 [1]. Tamoxifen, a selective estrogen receptor (ER) modulator, is the gold standard for treating ER-positive breast cancer. However, roughly 75% of all breast cancer cases are ER-positive and up to 30% of ER-positive breast cancer patients receiving tamoxifen treatment relapse due to tamoxifen resistance (TamR) [2]. Multiple mechanisms have been shown to contribute to TamR. Patients with higher levels of ER correlate with better response to tamoxifen therapy, and lack of ER expression contributes to TamR [3]. The TGF-β signaling pathway can promote various kinds of chemoresistance through enhancing cell proliferation, migration, and invasion by inducing epithelial–mesenchymal transition [4]. MicroRNAs (miRNAs) are also involved in TamR. For example, miR-200 can sensitize tamoxifen response through directly targeting multiple TGF-β signaling molecules [5].

Long noncoding RNAs (lncRNAs) are a class of newly identified noncoding transcripts that are usually composed of more than 200 nucleotides [6]. Recently, lncRNAs have been shown to be a novel class of regulatory molecules in drug resistance. HOX antisense intergenic RNA (HOTAIR) is transcribed from the antisense strand of the HOXC locus and is a most upregulated lncRNA in breast cancer. HOTAIR has been found to be able to promote multiple drug resistance events. In human lung adenocarcinoma cells, HOTAIR contributes to cisplatin resistance via downregulation of p21WAF1/CIP1 expression [7]. During the preparation of this manuscript, HOTAIR was reported as a promoter for TamR through enhancing...
ER signaling in breast cancer [8]. Besides, breast cancer antiestrogen resistance 4 (BCAR4), urothelial carcinoma-associated 1 (UCA1), lncRNA-ROR (ROR, regulator of reprogramming), colon cancer associated transcript 2 (CCAT2), and DSCAM-AS1 are also reported to enhance TamR [9-13]. However, the overall roles of lncRNAs in TamR remain to be discovered.

In this work, we first used a bioinformatic approach to establish the connection of lncRNA with TamR-related mRNAs and miRNAs by starBase v2.0 [14]. Among the 16 tested lncRNAs, LINC00894-002 (ENST00000444489), a lncRNA derived from X chromosome, shows the most sophisticated pattern in network predication and is the most downregulated lncRNA in tamoxifen-resistant MCF-7 cells (MCF-7/TamR) versus sensitive MCF-7/WT cells. Further studies showed that LINC00894-002 possessed an inhibitory effect on TamR. Moreover, LINC00894-002 is directly upregulated by ERα and positively regulates the expression levels of miR-200a-3p and miR-200b-3p, which inhibits the downstream TGF-β2-ZEB1 signaling pathway. To the best of our knowledge, this is the first reported inhibitory lncRNA against TamR. Our data suggest that the inhibitory role of LINC00894-002 may be achieved via the miR-200-TGF-β2-ZEB1 signaling pathway.

MATERIALS AND METHODS

Cell lines, cell culture, and reagents. The authentication of MCF-7/WT and MCF-7/TamR cells by STR profile analysis was performed by a third party (Genewiz, China) (data are available upon request). MCF-7/TamR cell line was derived from its parental cell line MCF-7/WT continuously exposed to tamoxifen (1 μM) (Sigma, USA) [15]. Both cell lines were cultured in RPMI 1640 (Invitrogen, USA) medium with 2 g/liter NaHCO3 (Sigma) and subsequently washed five times with 1% acetic acid to enhance TamR [9-13]. Moreover, MCF-7/TamR cells were cultured with 1% penicillin-streptomycin (WISENT, China). To maintain the resistance of MCF-7/TamR, it was continuously cultured with 1 μM tamoxifen.

Sulforhodamine B (SRB) assay. The SRB assay was modified according to the method described by Pauwels et al. [16]. Briefly, 3000 cells/well were seeded in 96-well plates for 24 h before receiving vehicle or tamoxifen treatment. After 96 h, the culture medium was aspirated, and the remaining cells were fixed with 10% trichloroacetic acid (TCA) at 4°C (200 μl/well) for 1 h. The cells were then washed five times with 100 μl deionized water and left to air-dry at room temperature. The cells were then stained with 100 μl 0.4% SRB (Sigma) at 37°C for 30 min and subsequently washed five times with 1% acetic acid to remove unbound stain. Lastly, the plates were dried at 37°C, and the bound protein stain was solubilized with 100 μl of 10 mM unbuffered Tris-base on a shaker for 30 min. Afterwards, the 96-well plates were transferred to a CLARIOstar microplate reader (BMG LABTECH, Germany) to detect optical density (OD) at 515 nm. The formula below was used to calculate the cell growth rate [17]:

\[
\text{cell growth rate (\%) = } \frac{\text{mean OD}_{\text{tamoxifen}} - \text{mean OD}_{\text{vehicle}} - \text{mean OD}_{24\text{h}}}{\text{mean OD}_{\text{24h}}} \times 100%.
\]

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen) followed by DNase I (Thermo Scientific, USA) treatment. The RNAs were then reverse-transcribed with HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, China), followed by qPCR on a LightCycler® 96 System (Roche, Switzerland) using SYBR Green master mix (Vazyme) and specific primers (GENERAL BIOSYSTEMS, China) (primers are listed in Table S1; see Supplement to this paper on the site of the journal http://protein.bio.msu.ru/biokhimiya and Springer site Link.springer.com). MiRNAs were reverse-transcribed and detected by the All-in-One™ miRNA qRT-PCR Detection System (GeneCopoeia, USA) following the manufacturer’s instructions.

Nucleus–cytoplasm segregation experiment. MCF-7/TamR cells were lysed in 500 μl RSB-100 buffer (100 mM Tris-HCl, 100 mM NaCl, 2.5 mM MgCl2, 40 μg/ml digitonin, pH 7.4) on ice for 8 min and then centrifuged at 2000g for 8 min. The supernatant fraction was collected as the cytoplasm fraction. The pellet was then resuspended in 300 μl RSB-100T (RSB-100 with 0.5% Triton X-100) on ice for 8 min and centrifuged at 2000g for 8 min; the precipitate was collected as the nucleus fraction. The cytoplasm and nucleus fractions were used for RNA extraction following procedures described above.

Western blotting. Proteins were extracted from cultured cells using TRIzol reagent following the manufacturer’s instructions. Equal (by protein content) aliquots from each sample were separated by SDS-PAGE and transferred to PVDF membranes (Merck, Germany). Subsequently, the membranes were blocked in 5% milk/1× TBS for 1 h and then incubated with the primary antibody against GAPDH (Proteintech, USA) overnight. After a thorough wash, the membranes were incubated with rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody (Proteintech) for 1 h. Finally, after washing, bands were visualized by ECL detection reagent (Thermo Scientific) on a UVP device (Analytik Jena AG, Germany).

Antisense oligonucleotide (ASO) transfection. MCF-7/TamR cells (3·104 per well) were seeded in 6-well plates for 12 h and then starved for 12 h before transfection. The ASO specifically targeting LINC00894-002, Inc-ATB, or the negative control (Ribobio, China) were transfected at

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