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LINC00680 modulates docetaxel resistance in breast cancer via the miR-320b/CDKL5 axis

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Abstract

Introduction: Increasing evidence has indicated that LINC00680 represents an oncogenic factor in cancer; however, the mechanism by which LINC00680 contributes to breast cancer (BC) remains unknown.

Methods: A dual-luciferase reporter assay was used to explore the relationship between LINC00680, miR-320b, and cyclin-dependent kinase 5 (CDKL5). A CCK-8 assay and transwell assay were utilized to evaluate the proliferation and invasion in docetaxel-resistant BC cells, respectively.

Results: LINC00680 and CDKL5 protein levels were both upregulated when induced by different concentrations of docetaxel. LINC00680 knockdown decreased the expression level of drug resistance-related genes, proliferation, and invasion of BC cells. Bioinformatics prediction and dual-luciferase assays revealed that miR-320b targeted the 3′-untranslated regions (UTR) of both LINC00680 and CDKL5, suggesting that the modulation of LINC00680 on CDKL5 occurred via sequestering miR-320b.

Conclusion: Overall, this study highlights the important role of LINC00680 in docetaxel resistance through the miR-320b/CDKL5 pathway and provides a novel therapeutic strategy for BC drug resistance.

Keywords

LINC00680, miR-320b, docetaxel resistance, CDKL5, ceRNA network

Introductions

Breast cancer (BC) is the most common cancer in women worldwide and the leading cause of cancer-related mortality among women.[1] Despite remarkable progress in the treatment of BC over the past decade, mortality from this cancer type is gradually increasing due to frequent chemotherapeutic resistance and tumor metastasis.[2,3] Importantly, in clinical practice, taxanes, including paclitaxel and docetaxel, are commonly accepted as chemotherapeutic agents for BC;[4] however, patient resistance to docetaxel remains a persistent problem.

In recent years, several genes have been found to be associated with docetaxel resistance. For example, differential expression of the ATP-binding cassette subfamily B member 1 (*ABCB1*) gene is a putative biomarker in docetaxel-resistance cancers,[5,6] whereas permeability glycoprotein (P-gp) acts as an ATP-dependent efflux pump and reduces docetaxel concentration by expelling the drug.[7] Furthermore, cyclin-dependent kinase-like 5 (CDKL5) deficiency or mutations could cause a distinct disorder. Additionally, Jiang et al.[8] found that CDKL5 could promote the

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progression and β-lapachone-resistance of glioma. Robin et al.[9] presented CDKL5 as a prognostic marker for glioblastoma, and Masahiro et al.[10] suggested the CDKL5 peptide can be used for cytotoxic T-lymphocyte-mediated immunotherapy. However, there has been no study to date that has examined the role of CDKL5 in the progression of chemoresistance in BC.

Long non-coding RNAs (lncRNAs) are a type of RNA with a length of over 200 nucleotides and usually influence mRNA generation and expression.[11] Previous studies have implicated lncRNAs in chemotherapy resistance, and lncRNAs generally regulate gene expression at the post-transcriptional level via sponging microRNAs[12] and modulating transcriptional gene expression.[13,14] For example, SNHG7 was shown to mediate both the chemoresistance of BC and cancer stemness by sponging miR-34a,[15] and actin filament-associated protein antisense RNA 1 (AFAP1-AS1) was reported to induce trastuzumab resistance through associating with AU-binding factor 1 (AUF1) and promoting epidermal growth factor receptor 2 (ERBB2) translation.[16] In a previous study, LINC00680 was first reported as a protective biomarker and independent prognostic indicator of soft tissue sarcoma,[17] and then it was reported to promote the progression of non-small cell lung cancer and glioblastoma cells.[18,19] Recently, the functional roles of LINC00680 in hepatocellular carcinoma stemness and chemoresistance have been explored; however, the studies investigating the underlying mechanism of LINC00680 in BC progression and docetaxel resistance are limited.

In the current study, we examined the interaction of LINC00680 and CDKL5 and their role in the regulation of chemoresistance of BC. Our results demonstrate the novel role of LINC00680 as a metastasis-promoting molecule in BC by upregulating CDKL5, highlighting the clinical potential of LINC00680 as a novel therapeutic target for BC.

Materials and methods

Cell lines and culture

Human breast cancer cell lines MCF-7 (Accession Number: SCSP-531) and MDA-MB-231 (Accession Number: SCSP-5043) were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). The derived docetaxel-resistant variants of MCF-7 cells (MCF-7/R) and MDA-MB-231 (MDA-MB-231/R) were generated in a stepwise manner by exposure to increasing doses of docetaxel as recently described.[20] The chemoresistant BC cells were maintained in a drug-free medium for 2 weeks before subsequent experiments were performed to avoid the influence of a toxic insult, and parental cells were cultured unexposed to docetaxel as a control for all experiments.

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U/ml penicillin, and 100 μg/mL streptomycin (both purchased from Beyotime Ltd, Shanghai, China), and incubated at 37°C in a humidified incubator with 5% CO_2.

Drug resistance and proliferation analyses

Drug cytotoxicity and proliferation were measured by Cell Counting Kit-8 (CCK-8) assays in vitro. Approximately 5,000 cells suspended in a 100-μl culture medium were plated in 96-well plates and incubated with increasing concentrations of docetaxel. To calculate the drug lethality to 50% of the cells (IC_{50}), the proliferative ability of untreated cells was used as the 100% standard. After incubation for 48 h, 10 μl of CCK-8 reagents were added, and the absorbance at 450 nm was detected according to the manufacturer's instructions using a Multiskan FC (Thermo Fisher Scientific, Waltham, MA, USA).

RNA interference and transfection

The RNA interference sequence for LINC00680 (Si-LNC), miR-320b (miR-320b inhibitor), CDKL5 (Si-CDKL5), and corresponding negative controls (Scramble and miR-NC) were designed and synthesized by Gene pharma company (Shanghai, China). siRNA sequence targeting was as follows: Si-LNC, forward oligo, 5'-UAUAGUUCAAGUCAUAAA CUG-3', reverse oligo, 5'-GUUUAUGACUUGAACUAU AGG-3'; Si-CDKL5, forward oligo, 5'-UCACAUAUUU-GUUCUAAUCAU-3'; reverse oligo, 5'-GAUUAGAA-CAAAUAUGUGAAA-3'. Plasmid vector pcRNA3.1-CDKL5 (CDKL5) was constructed by RIBOBIO (Guangzhou, China), and pcDNA3.1 empty vector was used as a negative control. Interfering RNAs were transfected into cells in 6-well plates using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR) assays

RNA was isolated from cells using TRIZol reagent (Thermo Fisher Scientific) and quantified using a NanoDrop 2000 spectrophotometer. Then 2 μg of total RNA was reversed transcribed to cDNA using SuperScript™ IV One-Step RT-PCR System (Thermo Fisher Scientific). SYBR Green PCR Master Mix (Thermo Fisher Scientific) was performed to examine the expression of genes using an ABI7500 system (Applied Biosystems, Foster City, CA, USA). The primers used in this study are shown in Table 1. The relative levels of gene expression were expressed relative to glyceraldehyde-

Table 1. Primers for qRT-PCR assay.

Gene Name	Primers (5' to 3')	
LINC00680	Forward	AGATGGTGAGAACTGGTCTGA
—	Reverse	GGGCTCTGGGTCTGAATCTT
miR-320b	Forward	GATGCTGAAAAGCTGGGTTG
—	Reverse	TATGGTTGTTCTGCTCTCTGTCTC
CDKL5	Forward	CTGGGGAAGGTAAAGCGGC
—	Reverse	TTAAATGACTCCCCCGCCGA
MDR1	Forward	GGCTACATGAGAGCGGAGGA
—	Reverse	GGAATGTTCTGGCTTCCGTTG
MRP5	Forward	TTGCTACGTGAGTGTACGCC
—	Reverse	GATGTGAGGACTGGCTGGTT
LRP5	Forward	GTTACACTGGAGAGAGCAGCAT
—	Reverse	CCTCTGTCCTCTCATCCTTCA
GAPDH	Forward	ACAGTCAGCCGCATCTTCTT
—	Reverse	GACTCCGACCTTCACCTTCC
SncU6	Forward	CTCGCTTCGGCAGCACA
—	Reverse	AACGCTTCACGAATTTGCGT

3-phosphate dehydrogenase (*GAPDH*) or *SncU6* using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Cells were lysed in RIPA lysis buffer (Beyotime) supplemented within a protease inhibitor cocktail (Roche Applied Science, Indian polis, IN, USA). The samples were incubated at 4°C for 30 min and then centrifuged at 12,000 × g for 15 min at 4°C. The protein supernatant (total 30 μg proteins) was then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The PVDF membrane was incubated with primary antibodies against CDKL5 and β-actin (both purchased from Abcam, Cambridge, CA, USA) at 4°C for 18 h. The membrane was then incubated with a second antibody (horseradish peroxidase-conjugated goat anti-rabbit or horseradish peroxidase-conjugated goat anti-mouse) at room temperature at 25°C for 2 h. Finally, the blots were developed using an ECL chemiluminescence detection kit (Thermo Fisher Scientific).

Cell invasion assay

Briefly, cells (1×10^4 cells per well) were seeded and suspended in a medium containing 1% FBS. The cells were then plated into the upper chamber of a transwell plate, which was pre-coated with Matrigel, and the lower chamber was filled with DMEM medium with 10% FBS. After 24 h of incubation, the non-invaded cells on the upper surface of the membrane were removed using cotton. The cells that invaded through the membrane were washed with phosphate-buffered saline and then fixed with methanol for 20 min at 25°C. The cells were stained with 0.1% crystal violet. The number of invaded cells in five randomly selected fields of each group was counted. All experiments were performed in triplicate.

RNA immunoprecipitation (RIP) assay

An RIP assay was performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA) according to the manufacturer's instructions. Briefly, the cell lysate prepared from 2×10^7 MCF-7 cells was incubated with magnetic beads conjugated with negative control normal mouse IgG or human anti-Ago2 antibody (Millipore). The immunoprecipitated RNAs were then extracted and detected by qRT-PCR assays to confirm the enrichment of binding targets.

Dual-luciferase reporter assay

The 3′-UTR of *LINC00680* and *CDKL5* mRNA containing miR-320b binding sites were amplified and cloned into the pGL3-Basic luciferase vector (Promega, Madison, WI, USA). Then LINC00680 and CDKL5 mRNA, wild-type or mutant sequences, were synthesized and transfected into HEK-293T cells with miR-320 b mimics or negative controls using Lipofectamine™ 2000 reagent (Thermo Fisher Scientific). After 24 h of transfection, the luciferase activity was measured using Dual-Luciferase Reported System compared to Renilla luciferase (Promega).

Statistical analysis

Data are presented as the mean ± SD. The statistical analyses were performed using SPSS version 19.0 (IBM, Chicago, IL, USA) and charted using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). A one-way analysis of variance was used for differences among multiple groups and a Student's *t*-test was used for comparing between two groups. Furthermore, $p < 0.05$ was considered statistically significant.

Results

Identification of docetaxel-resistant BC cells

To compare the IC50 of docetaxel to parental and docetaxel-resistant cells, the sensitivities of these cells to various concentrations of docetaxel were determined by a CCK-8 assay. The IC50 of docetaxel in MCF-7 and MCF-7/R cells was 0.75 and 83.4 μM, respectively (Figure 1(a), Figure 1(b)). Consistently, the cell viability of docetaxel-treated MDA-MB-231/R cells was significantly higher

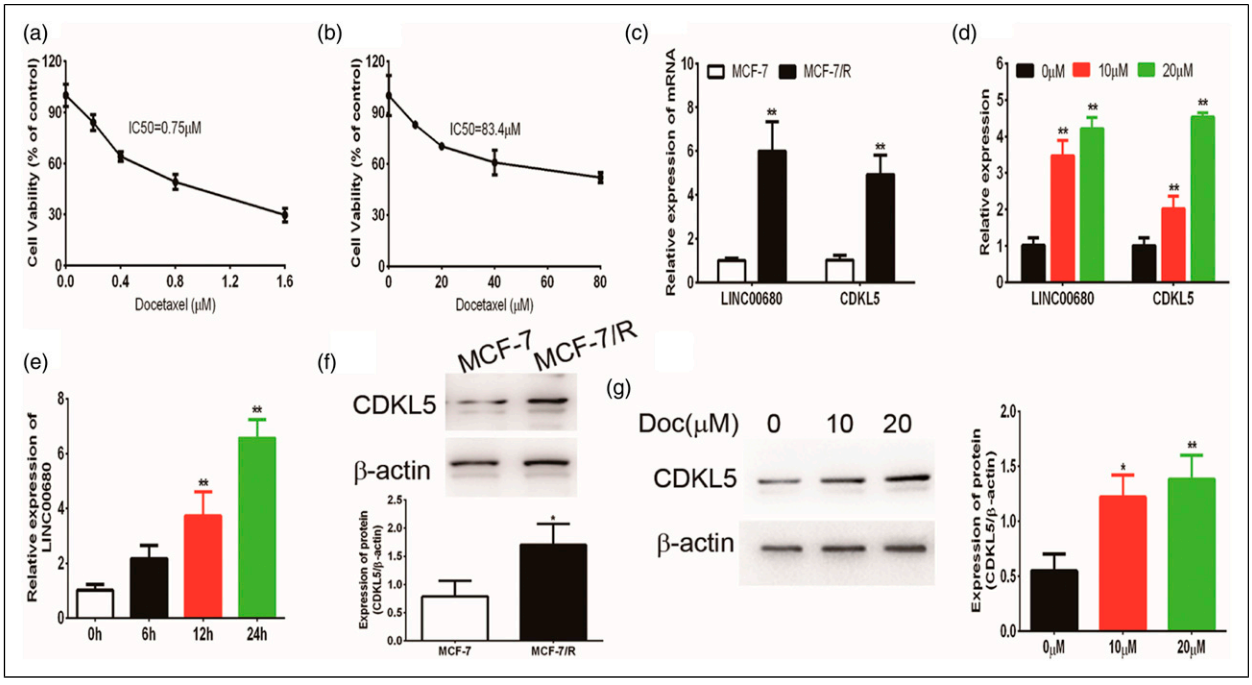

Figure 1. LINC00680 and CDKL5 are both upregulated in MCF-7/R cells. (a–b) CCK-8 assay was performed to assess the IC50 value of MCF-7 and MCF-7/R cells, respectively. (c) qRT-PCR assay indicated the levels of *LINC00680* and *CDKL5* in MCF-7 cells and MCF-7/R cells. (d–e) qRT-PCR assay indicated the levels of *LINC00680* and *CDKL5* in MCF-7/R cells incubated with different dose of docetaxel or different processed time. (F) Western blot was performed to measure the expression of CDKL5 protein in MCF-7 and MCF-7/R cells. (g) Expression of CDKL5 in MCF-7/R cells incubated with different dose of docetaxel. Data was expressed as mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the MCF-7 group or Control group (cells treated withdraw docetaxel). LINC00680 silencing inhibited cell proliferation and invasion of docetaxel-resistant BC cells by suppressing CDKL5 expression.

than that of docetaxel-treated parental MDA-MB-231 cells (Supplement Figure S1). Thus, these results imply that MCF-7/Doc and MDA-MB-231/Doc are both docetaxel-resistant cell lines.

LINC00680 and CDKL5 proteins were upregulated in docetaxel-resistant BC cells

To investigate the expression level of LINC00680, qRT-PCR and western blot assays were performed in docetaxel-resistant BC cells. Our results showed that the expression of LINC00680 and CDKL5 was significantly upregulated in MCF-7/Doc cells and MDA-MB-231/Doc cells as compared with MCF-7 and MDA-MB-231 cells (Figure 1(c), Figure 1(f), and Supplement Figure S1). Additionally, the expression of LINC00680 and CDKL5 was dose-dependent in MCF-7/Doc cells, and LINC00680 expression was notably increased after 12 h of docetaxel incubation (Figures 1(d), (f), and (g)). Taken together, these data suggested that LINC00680 and CDKL5 were both upregulated in MCF-7/Doc cells, and LINC00680 and CDKL5 were closely associated with docetaxel resistance in BC cells.

To further investigate the functional roles of LINC00680 in chemoresistance, MCF-7/Doc and MDA-MB-231/Doc cells were transfected with Si-LNC or Scramble negative interfering RNAs. Both the transcript and protein levels of LINC00680 and CDKL5 were dramatically repressed in LINC00680-silenced MCF-7/Doc cells (Figure 2(a) and Figure 2(b)) and MDA-MB-231/Doc cells with respect to control cells (Supplement Figure S2). Additionally, the deficiency of LINC00680 inhibited cell proliferation and invasion in MCF-7/Doc cells, whereas CDKL5 overexpression facilitated cell proliferation and invasion in MCF-7/Doc and MDA-MB-231/Doc cells (Supplement Figure S2). In addition, silencing of LINC00680 weakened the proliferation and invasion of MCF-7/Doc cells, which could be abolished by the introduction of CDKL5 (Figure 2(c) and (d)). In summary, these results revealed that LINC00680 downregulation inhibits cell proliferation and invasion of docetaxel-resistant BC cells by suppressing CDKL5 expression.

In present experiments, we verified that the protein levels of LINC00680 and CDKL5 were upregulated in MCF-7/Doc cells when treated with docetaxel. As such, we suspected that LINC00680 may function as a competing endogenous RNA to repress some microRNAs. Bioinformatics analysis showed

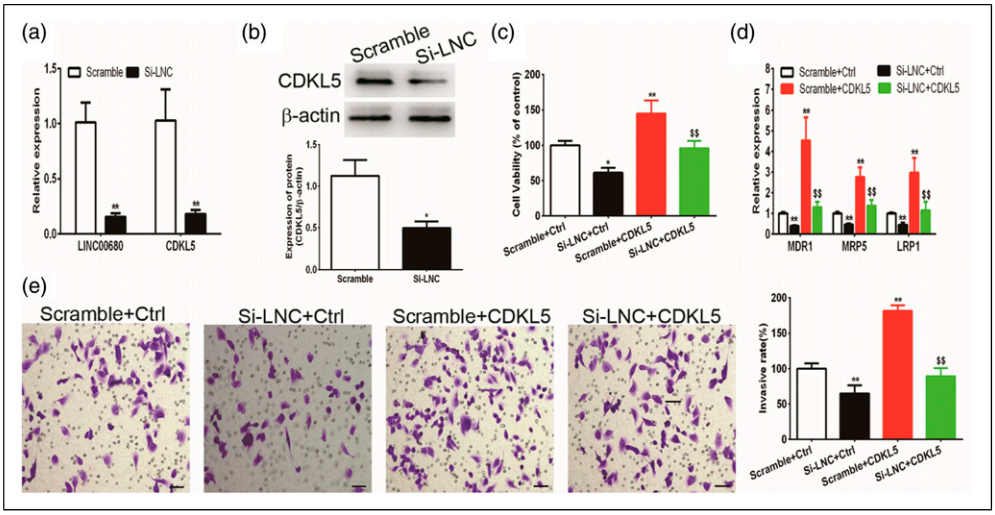

Figure 2. LINC00680 silencing inhibits cell proliferation and invasion of docetaxel-treated MCF-7/R cells by suppressing CDKL5 expression. (a–b) qRT-PCR and Western blot showed *LINC00680* knockdown inhibited *LINC00680* and *CDKL5* expression MCF-7/R cells. (c) CCK-8 assay was performed to detect the cell proliferation of MCF-7/R cells, which were co-transfected with Si-LNC, pcDNA3.1-CDKL5 (CDKL5) or corresponding negative control (Scramble or Ctrl). (d) qRT-PCR was performed to assess the mRNA levels of drug resistance related genes in modified MCF-7/R cells. (e) Transwell cell invasion assay was performed to assess the capacity invasive in modified MCF-7/R cells. Data was expressed as mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, compared with Scramble group; $^{\$\$}p < 0.01$, compared with the Si-LNC + Ctrl group. LINC00680 modulated CDKL5 expression through competitively binding miR-320b.

that miR-320b shared with LINC00680 3'-untranslated region (UTR), and miR-320b was predicted to bind at the 931–937 position in the CDKL5 3'-UTR. The dual-luciferase reporter results indicated that co-transfection with miR-320b and the wild-type 3'UTR of CDKL5 (or wild-type LINC00680) decreased the firefly luciferase activity, whereas which was not affected by co-transfection of miR-320b/CDKL5 3′UTR mutant or miR-320b/mutant-type LINC00680, respectively (Figures 3(a), (b), (e) and (f)). Furthermore, the RIP assay indicated that compared to the IgG control, an increased enrichment of LINC00680 and miR-320b in Ago2 precipitated pellet (Supplement Figure S3).

Moreover, the expression of miR-320b was found to be significantly downregulated in MCF-7/Doc cells as compared to MCF-7 cells, and the gene and protein of CDKL5 were obviously repressed in MCF-7/Doc cells transfected with miR-320b (Figures 3(b) and (c)). Furthermore, the inhibitory effect of miR-320b upregulation on CDKL5 gene and protein expression was rescued by LINC00680 overexpression (Figures 3(h) and (i)). Collectively, our results indicated that LINC00680 functions as a competing endogenous RNA to repress miR-320b, which controls its downstream target CDKL5.

In the present study, we reported the involvement of the LINC00680/miR-320b/CDKL5 axis in BC tumorigenesis, and then we performed functional experiments to confirm this. The results showed that the levels of multidrug resistance-related genes (MDR1, MRP5, and LRP1) decreased in CDKL5-knockdown or LINC00680-silenced cells, while the expressions of these genes increased after transfection with an miR-320b inhibitor in LINC00680-silenced groups. Additionally, CDKL5 knockdown or LINC00680 knockdown suppressed the proliferation rate and invasion capacity, which could be reversed by the introduction of an miR-320b inhibitor. Therefore, the above data highlighted the role of the LINC00680/miR-320b/CDKL5 signaling pathway in BC proliferation, invasion, and docetaxel resistance Figure 4.

Discussion

The main problem in cancer therapy is the inherent resistance to chemotherapy, which causes disease, relapse, and metastasis, and remains the main obstacle to cancer therapy. The molecular mechanism of chemoresistance includes various factors, such as tumor suppressor gene, mitochondrial alteration, DNA repair, and cancer stemness.[21] As a common anti-tumor drug, docetaxel plays a vital role in the inhibition of protein and nucleic acid synthesis. However, the mechanism of docetaxel resistance is still unknown. Therefore, identifying molecules or genes involved in BC chemoresistance has great significance in the treatment of BC.

Although several studies have recently demonstrated the modulating role of lncRNAs in tumor chemosensitivity,[22,23] there are limited studies regarding the role LINC00680 plays

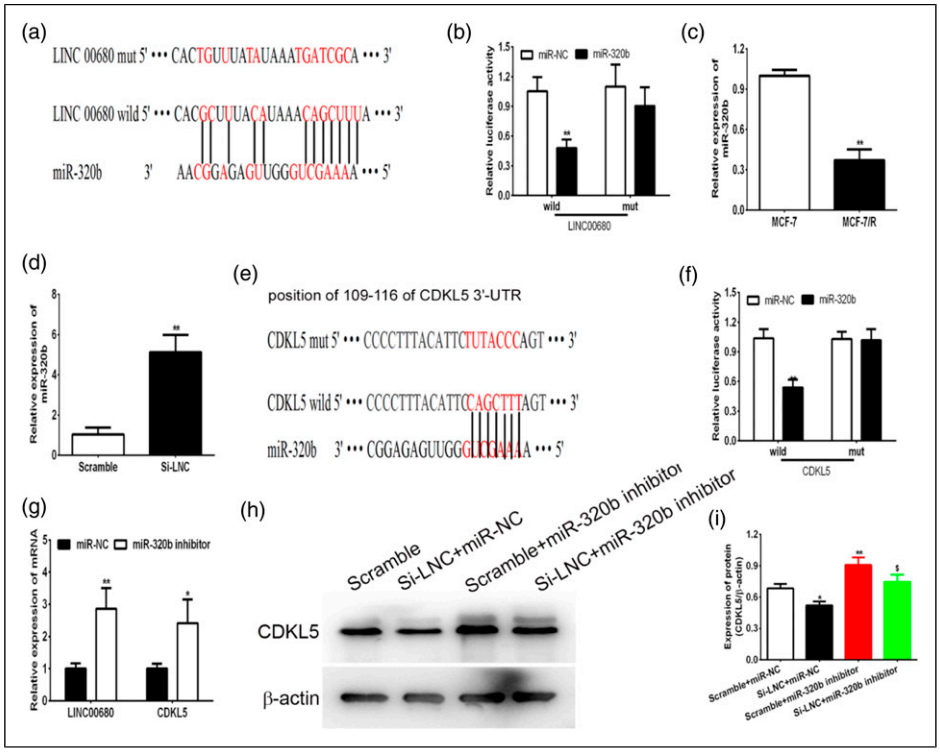

Figure 3. LINC00680 modulates CDKL5 expression via competitively binding miR-320b. (a) Bioinformatics tools showed the complementary binding sequence of miR-320b and 3′-UTR of *LINC00680* wild type and mutant type. (b) Dual-luciferase reporter assay showed the luciferase vitality within miR-320b/vector and 3′-UTR of *LINC00680* mutant or wild type. (c) The levels of *miR-320b* in MCF-7/R cells transfected with si-LINC00680 detected by qRT-PCR assay. (d) Bioinformatics tools showed the complementary binding sequence of *miR-320b* and *CDKL5* 3'-UTR. (e) Dual-luciferase reporter assay showed the luciferase vitality within miR-320b/vector and 3′-UTR of *CDKL5* mutant or wild type. (F–I) Western blot analysis were performed to measure the protein expression of CDKL5 in MCF-7/R cells transfected with Si-LNC or/and miR-320b inhibitor. Data was expressed as mean ± SD, * $p< 0.05$, ** $p < 0.01$, compared with the Scramble+miR-NC group; $^{\$}p<0.05$, compared with Si-LNC + miR-NC group. The associated role of LINC00680, miR-320b, and CDKL5 on BC proliferation, invasion, and docetaxel resistance.

in chemoresistance. According to the GEPA dataset, LINC00680 is not dysregulated in BC cells (Supplement Figure S3); however, compared with parental BC cells, LINC00680 was remarkably upregulated in docetaxel-resistant BC cells (Figure 1, Supplement Figure S1). Further, in vitro experiments demonstrated that LINC00680 silencing not only promoted docetaxel sensitivity in MCF-7/Doc cells (Supplement Figure S3), but also inhibited cell proliferation, invasion, and reduced the expression of MDR genes as well as CDKL5.

Based on the above findings, we further analyzed the underlying molecular mechanism by which LINC00680 modulated BC chemosensitivity. Current data support the role of lncRNAs generally functioning as competing endogenous RNAs of miRNAs, which affects BC progression via regulating mRNA expression.[24,25] Bioinformatics analysis suggests that LINC00680 has putative binding sites for members of the miR-320 family, including miR-320a, miR-320b, miR-320c, and miR-320d (data was not shown). Among these miRNAs, no study has shown the role of miR-320b on chemoresistance in BC. Thus, we selected miR-320b for further study. Consistently, bioinformatics analysis indicated that CDKL5 is the target gene of miR-320b. Fortunately, a luciferase reporter and RIP assay (Figure 3, Supplement Figure S3) both indicated that LINC00680 is very likely to exert its function via sponging miR-320b, thereby controlling its downstream target CDKL5.

In humans, mutations in the *CDKL5* gene are associated with neurodevelopmental disorders,[26] but little has been revealed on its role in cancer. Recent reports have identified that CDKL5-dependent glycogen synthase kinase 3β (GSK3β) regulatory mechanisms control cell apoptosis,[27] and CDKL5 can modulate glioma chemoresistance through the PI3K/AKT axis,[8] which has been shown to mediate critical physiological functions, including cell death.[28] Thus, some novel drugs, which sensitize cells to docetaxel treatment and allow them to overcome chemoresistance, are designed based on their ability to block the apoptotic

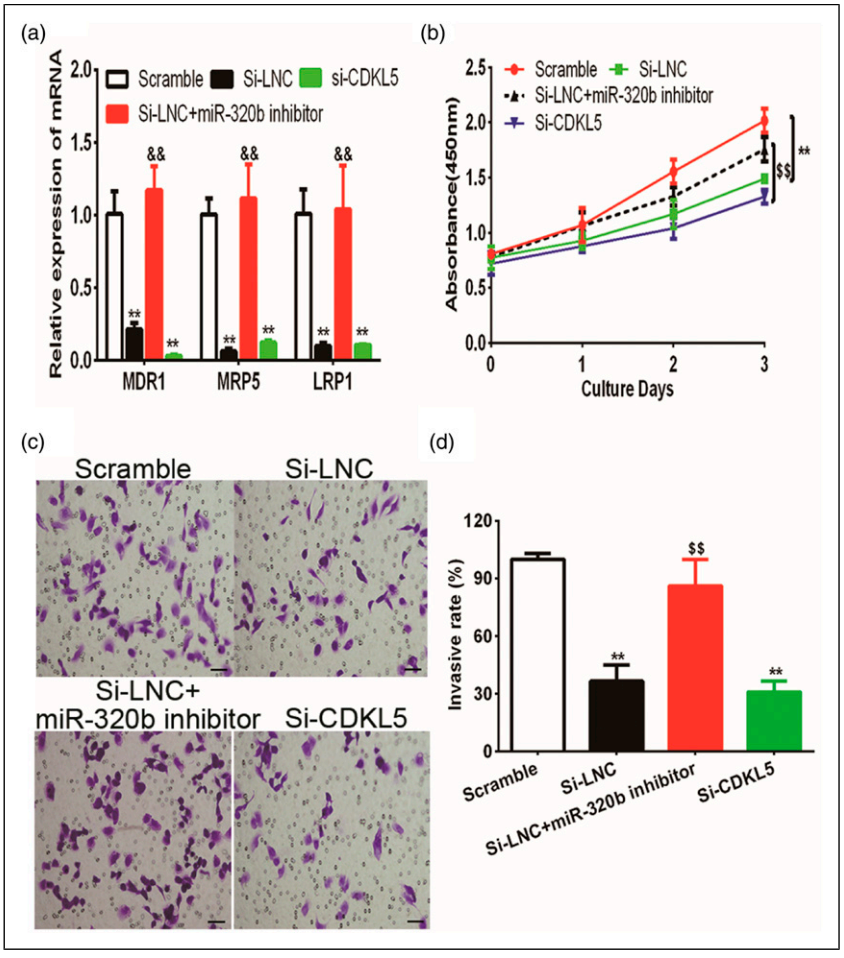

Figure 4. Associated role of LINC00680, miR-320b and CDKL5 on breast cancer proliferation, invasion and docetaxel resistance. (a) qRT-PCR was performed to assess the mRNA expression of drug resistance related gene in MCF-7/R cells transfected with Scramble, Si-LNC, Si-LNC within miR-320b inhibitor or Si-CDKL5 vector. (b) CCK-8 assay indicated the proliferation vitality of MCF-7/R cells with modified vector. (c) Transwell invasion assay showed the invasive ability of MCF-7/R cells transfected with modified vector. Data was expressed as mean ± SD, $^{**}p < 0.01$, compared with the Scramble group. $^{\$\$}p < 0.01$, compared with the Si-LNC group.

cascade.[29,30] Therefore, we assumed that the LINC00680/CDKL5 axis might influence docetaxel resistance in BC cells through regulating apoptotic signaling; however, this hypothesis needs to be confirmed by further investigation. Our present study had limitations. For example, the effect of the LINC00680/miR-320b/CDKL5 axis in the regulation of docetaxel resistance in BC cells needs to be further clarified in animal models, and the results should also be verified in other BC cell lines.

Conclusions

Consistent with previous findings in other cancers, our results revealed that LINC00680 promoted docetaxel resistance in BC cells by affecting cell proliferation and invasion via the miR-320b/CDKL5 axis. Taken together, the results indicated that LINC00680 may act as a regulator in the development of docetaxel resistance in BC cells, highlighting its clinical potential as a novel therapeutic target for BC.

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Author contributions

J.L. and J.K.: writing, literature research, data analysis, and statistical analysis; J.L., J.K., and C.L.Q.: literature search, clinical research, and data analysis;X.Z.: manuscript writing, literature research, study design, and manuscript review. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Ethics approval

Ethic approval for this study was obtained from the Ethics Committee of the Affiliated Hospital of Nantong University (ethic code: 2019-L156).

Informed consent

This study does not contain any clinical specimens or animals, so informed consent is not applicable.

Trial registration

This study did not contain any trial registration.

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Supplemental Material

Supplemental material for this article is available online.

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