

Upregulated Long Non-Coding RNA LL22NC03-N64E9.1 Promotes the Proliferation and Migration of Human Breast Cancer Cells by Silencing Kruppel-Like Factor 2 Expression

This article was published in the following Dove Press journal:
Cancer Management and Research

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Introduction: Recently, the significant regulatory effects of lncRNAs on the oncogenesis and growth of tumor have been demonstrated by an increasing number of research projects. A previous study showed that *LL22NC03-N64E9.1* could promote the development of colorectal cancer, especially via enhanced cell proliferation. Similarly, this lncRNA should have comparable functions in breast cancer (BC), which requires in-depth investigation. Therefore, this study was designed to explore the correlation of *LL22NC03-N64E9.1* with BC.

Methods: qRT-PCR was used to assess the relative expression of *LL22NC03-N64E9.1* in BC tissues. Cell viability examination and colony formation experiments were performed to investigate the role of *LL22NC03-N64E9.1* in BC cell's proliferation. Transwell assays were used to explore the effects of *LL22NC03-N64E9.1* on BC cell's migration. RNA immunoprecipitation, chromosome immunoprecipitation assay and rescue experiments were performed to analyze the association of *LL22NC03-N64E9.1* with target proteins and genes in BC cells.

Results: We identified that *LL22NC03-N64E9.1* is an oncogene, upregulated in BC, which was verified in a cohort of 48 pairs of BC tissues. Based on the loss-of-function experiments, silencing *LL22NC03-N64E9.1* expression significantly inhibited malignancy progression. In terms of the mechanism, *LL22NC03-N64E9.1* acted on the *enhancer of zeste homolog 2 (EZH2)* by direct binding, which promoted BC cell growth. Furthermore, in the promoters of *KLF2*, the trimethylation of *H3K27* could be regulated by *LL22NC03-N64E9.1* as the mediator.

Conclusion: Relying on the *LL22NC03-N64E9.1/EZH2/KLF2* pathway, the lncRNA *LL22NC03-N64E9.1* was significantly associated with BC development and could, therefore, be a potential therapeutic target to block BC growth.

Keywords: breast cancer, lncRNA, *LL22NC03-N64E9.1*, *H3K27me3*, *KLF2*

Background

Breast cancer (BC) has been reported as one of the most threatening diseases due to its high prevalence and mortality.^{1,2} In addition, there are also an increased number of reports which anticipate that the social burden caused by BC will continue to increase in most regions,³ for example China, in particular amongst the urban population.^{4,5} Recently, BC treatment strategies have been improved due to various

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remarkable achievements, although the overall survival is still expected to be further extended.^{6,7} Therefore, researchers are committed to identify and probe into novel therapeutic targets, specifically, oncogenes, thus the regulatory mechanisms at the genomic level still need to be explored.⁸

Non-coding sequences occupy 98% of the human genome and although they do not directly translate to proteins, they are designated to translate to various types of RNA transcripts, such as long non-coding RNAs (lncRNAs).^{9,10} The most definite characteristic of a lncRNA is the length, which is more than 200 nucleotides.¹¹ Although the existence of lncRNAs was previously mistaken for useless, accumulating evidence has revealed the clinical significance of lncRNAs as biomarkers and therapeutic targets.^{12,13} To our knowledge, several lncRNAs associated with BC prognosis have been identified, including *NEAT1*, which regulates cancer cell proliferation and invasion^{14,15} and *MALAT1*, which has been correlated to BC metastasis.¹⁶ However, there is a general consensus that multiple lncRNAs which have vital roles in the pathogenetic mechanisms of BC still need to be discovered, in particular the molecular details.

LL22NC03-N64E9.1, located at chromosome 22q11.1, has been reported as an oncogenic lncRNA and affects aggressive phenotypes in human lung and colorectal cancer.^{17,18} To investigate its relevance to BC, the expression profile of lncRNA *LL22NC03-N64E9.1* in human BC samples was compared with normal tissues. Subsequently, experiments were conducted to assess the association between *LL22NC03-N64E9.1*, and BC cell proliferation and migration. By rough estimation, 24% of lncRNAs could be physically associated with the *polycomb repressive complex 2 (PRC2)*.^{19,20} To clarify whether *LL22NC03-N64E9.1* could bind to the *PRC2* complex, *EZH2*, a core subunit of the *PRC2* complex, which catalyzed the trimethylation of *histone H3 at lysine 27 (H3K27me3)* was selected as the candidate target for the lncRNA.²¹ Finally, the downstream pathway regulated by altered *LL22NC03-N64E9.1* expression and *EZH2* was identified, and the underlying mechanisms of the lncRNA in BC cells were investigated.

Methods

Tissue Collection and Ethics Statement

The sample collection took place at Quanzhou First Hospital Affiliated to Fujian Medical University. After

the surgical procedures to remove primary BC, 48 BC tissue samples were acquired. The experiment protocol was reviewed and approved by the Ethics Committee of the Fujian Medical University (Fuzhou, Fujian, PR China), which followed the Declaration of Helsinki Principles. Accordingly, patient samples could only be used after obtaining signed informed consents.

Cell Lines and Cell Culture

MCF-7 and BT549, human cell lines of BC, were provided by the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, MO), 100 U/mL penicillin and 100 g/mL streptomycin was used for cell culture in a humidified incubator set at 37°C with 5% CO₂.

RNA Extraction and qRT-PCR Assays

All the experimental procedures were conducted following the manufacturers' instructions. Trizol reagent (Invitrogen) was used to extract total RNA from the cell culture. PrimeScript RT reagent Kit (TaKaRa, Dalian, China) was utilized to reverse transcribe 1 µg of extracted total RNA into 20 µL product containing cDNA. SYBR Premix Ex Taq (TaKaRa, Dalian, China) was applied to quantify *LL22NC03-N64E9.1* expression and its downstream targets, and the data were normalized to *GAPDH* level. The PCR primers had the following sequences: *LL22NC03-N64E9.1* (Forward) 5'- AAGCCATGTAAAGGGGGCT C-3'; *LL22NC03-N64E9.1* (Reverse) 5'- TGGTAGTCTG ACCATTCTGCAT-3'; *EZH2* (Forward) 5'- TGCACATCC TGACTTCTGTG-3'; *EZH2* (Reverse) 5'- AAGGGCATT CACCAACTCC -3'; *GAPDH* (Forward) 5'- GAAGAGA GAGACCCTCACGCTG -3'; *GAPDH* (Reverse) 5'- AC TGTGAGGAGGGGAGATTCAGT-3'; *kruppel-like factor 2 (KLF2)* (Forward) 5'- CTGCACATGAAACGGCACAT-3'; *KLF2* (Reverse) 5'-CAGTCACAGTTTGGGAGG GG-3'.

Cell Transfection

Lipofectamine 2000 (Invitrogen, USA) was the reagent for transfecting BC cell samples with the specific siRNAs. *LL22NC03-N64E9.1* siRNA and the siRNA of scrambled negative control (si-NC) were provided by Invitrogen. The siRNA sequences were as follows: *LL22NC03-N64E9.1* siRNA: UAGCUGGAGCAGUACAUCUCAAUU; si-NC: UUCUCCGAACGUGUCACGUTT; *EZH2* siRNA: GAGG UUCAGACGAGCUGAUUU. After transfection and 48h of

incubation, the transfected samples were usable for subsequent analyses.

Cell Proliferation Analysis

Cell viability was examined using the MTT kit (Sigma). To conduct the colony formation assay, transfected cell samples were placed in standard six-well cell culture plates and maintained in DMEM containing 10% FBS for 2 weeks. The medium was replaced every 5 days. After 2 weeks, samples were fixed with methanol and stained for 15 min with 0.1% crystal violet (Sigma) in PBS. The number of stained colonies was counted and compared to evaluate colony formation.

Transwell Assay

A 24-well chamber (Corning, NY, USA) was embedded with polycarbonate membrane of 8 μm pore size. To assess cell invasion and migration, the membrane was either coated with Matrigel (Becton Dickinson, NJ, USA) or not coated, respectively, and subsequently placed into the upper side of the chamber and incubated for 24h. Cells remaining on the upper side were gently wiped with cotton swabs, while cells that passed through the membrane onto the lower side were fixed and stained with 0.5% crystal violet. Five fields in each well were randomly selected, and the number of migrant cells were counted. Each test was conducted in triplicate.

RNA Immunoprecipitation (RIP) Assays

Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) was used according to manufacturer's protocol. The antibody against *EZH2* was provided by Millipore.

Chromatin Immunoprecipitation (ChIP) Assays

The EZ-CHIP KIT (Millipore, USA) was used to perform the CHIP assay. *EZH2* was provided by Abcam and the *H3 trimethyl Lys 27* antibody, Histone H3, and Acetyl-Histone H3 Lys27 were provided by Millipore. The ChIP primer sequences were: *KLF2* (Forward) 5'-ACGG GCTTATTGAGGTTGG-3' and *KLF2* (Reverse) 5'-GCC TGGGTGACAGAGGAGAC-3'. The immunoprecipitated DNA was quantitated by qPCR. The formula, $2^{[\text{Input Ct} - \text{Target Ct}]}$ $\times 100$ (%), was used to interpret the raw data. Each test was conducted independently in triplicate.

Statistical Analysis

Statistical analyses were performed using SPSS software version 17.0 (SPSS, IL, USA). The Student's *t*-test or the chi-square test was used to compare different data sets and determine the significance. The data were expressed as means \pm SD. Differences were considered as significant if $P < 0.05$. “*” indicates $P < 0.05$.

Results

LL22NC03-N64E9.1 is Upregulated in Human BC Tissue Samples

By interpreting the raw microarray data for human breast tissues obtained from the Cancer Genome Atlas (TCGA), the expression profile of *LL22NC03-N64E9.1* could be compared between the groups with or without BC. Compared with noncancerous tissues, *LL22NC03-N64E9.1* expression was obviously upregulated in BC tissues (Figure 1A). To further validate this result by qRT-PCR, we investigated 48 clinical BC samples and found that *LL22NC03-N64E9.1* expression was also upregulated

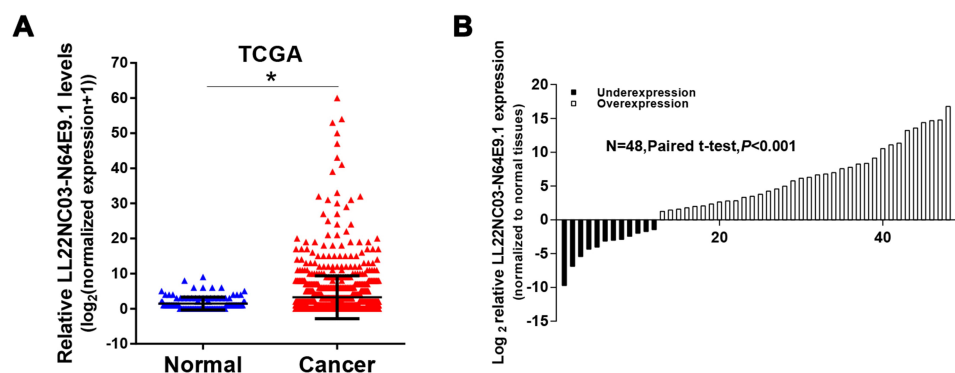


Figure 1 *LL22NC03-N64E9.1* expression is significantly upregulated in BC tissues. (A) Expression levels of *LL22NC03-N64E9.1* were compared between unpaired human BC tissues and normal tissues, using TCGA data (normal=33, tumor =285). (B) Expression levels of *LL22NC03-N64E9.1* in 48 BC tissues and adjacent normal tissues were assessed by qRT-PCR. * $P < 0.05$.

in the majority of BC samples (36 out of 48) compared with the adjacent noncancerous tissues ($P < 0.001$; Figure 1B).

LL22NC03-N64E9.1 Regulates BC Cell Proliferation and Migration

We examined whether *LL22NC03-N64E9.1* was functionally involved in BC progression and discovered that *LL22NC03-N64E9.1* expression was silenced after transfection with short interfering RNA (siRNA) in MCF-7 and BT549 cells (Figure 2A). To further assess the role of *LL22NC03-N64E9.1* in the BC cell phenotype, loss-of-

function assays were conducted. The findings provided by colony formation assay confirmed that cell colonies had significantly impeded growth in the *LL22NC03-N64E9.1* knockdown group, compared with the si-NC group in MCF-7 and BT549 cells (Figure 2B). In addition, MTT assay revealed that *LL22NC03-N64E9.1* knockdown significantly inhibited BC cell viability (Figure 2C). Transwell assay was conducted to determine whether *LL22NC03-N64E9.1* expression was associated with BC cell migration and the results showed that *LL22NC03-N64E9.1* knockdown significantly reduced the quantity of migratory cells among MCF-7 and BT549 BC cell lines,

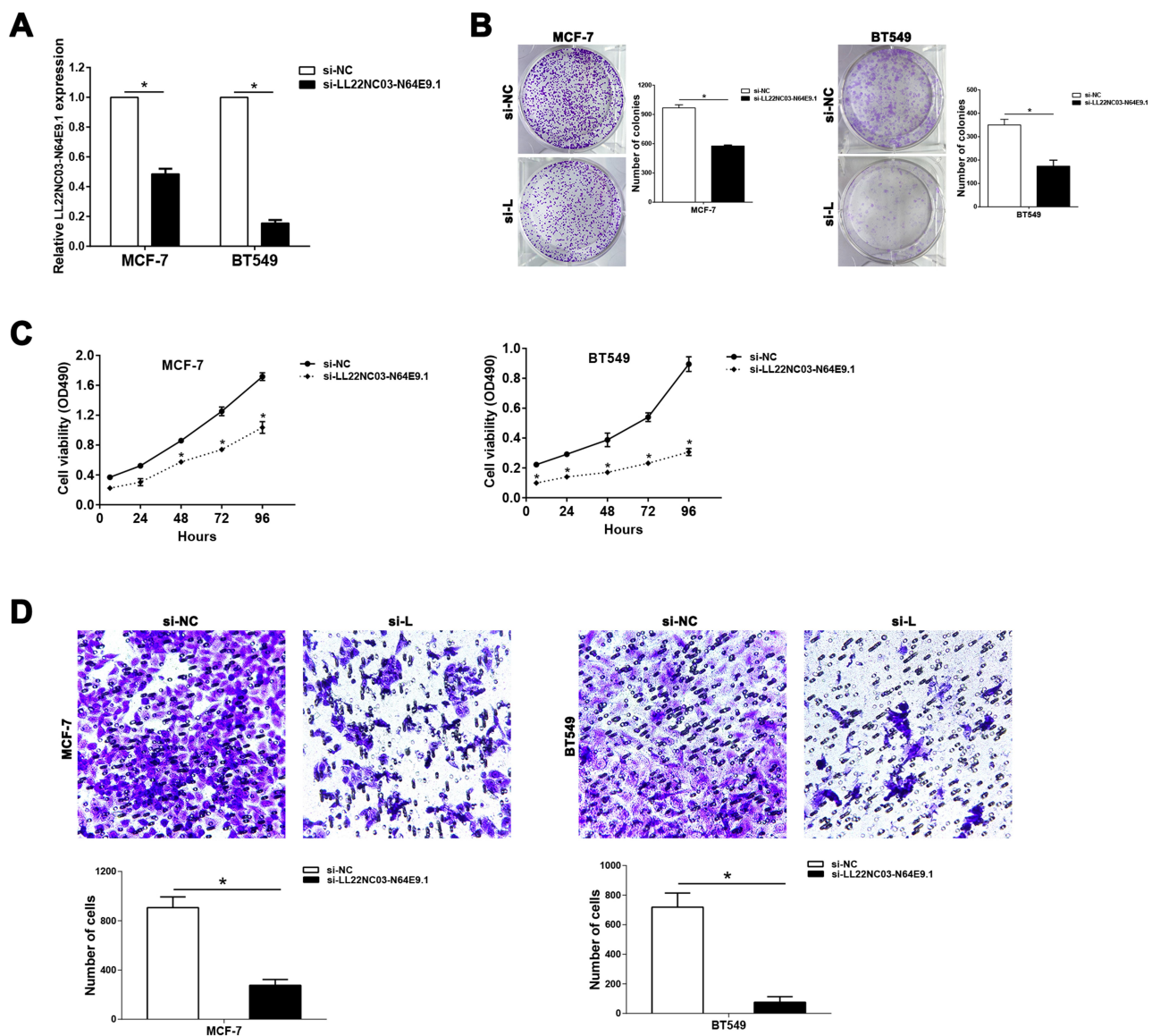


Figure 2 Knockdown of *LL22NC03-N64E9.1* represses the proliferation and migration of BC cells. (A) qRT-PCR results showed the effectiveness of *LL22NC03-N64E9.1* knockdown in MCF-7 and BT549 cells transfected with siRNA. (B and C) Colony formation (B) and MTT (C) assays were conducted to evaluate how the transfection with si-LL22NC03-N64E9.1 could affect BC cell proliferation. (D) Transwell assays were conducted to assess BC cell migration upon the transfection with si-NC or si-LL22NC03-N64E9.1. * $P < 0.05$.

compared with the si-NC group (Figure 2D). Overall, the silenced *LL22NC03-N64E9.1* expression reduced BC cell proliferation and migration.

LL22NC03-N64E9.1 Exerts Oncogene Function via Interacting with EZH2

Our subsequent task was to clarify whether *LL22NC03-N64E9.1* could bind to the PRC2 complex. RNA-protein interaction prediction (RPISeq) analysis determined the score for *LL22NC03-N64E9.1/EZH2* interaction, which was 0.8 with SVM classifier (data were not showed). With the probabilities of >0.5, the predictions were assigned to be positive, and accuracy for the predictions ranged from 57% to 99% for individual datasets of RNA-protein interactions.²² To verify the predictive results, RIP assays demonstrated the enrichment of endogenous *LL22NC03-N64E9.1* in the anti-*EZH2* fraction relative to the input and compared to the IgG fraction (Figure 3A). To measure the biological features of *EZH2* in BC, endogenous *EZH2* expression in MCF-7 cells was knocked down by siRNA (Figure 3B). Colony formation and MTT assays showed that down-regulated *EZH2* weakened the proliferation ability of MCF-7 cells (Figure 3C and D). Additionally, the

migration ability of BC cells was also strongly decreased due to *EZH2* knockdown (Figure 3E). These results summarize the oncogenic role of *EZH2* in promoting malignant BC cell proliferation and migration, which corresponds with the carcinogenesis of *LL22NC03-N64E9.1* in BC. Furthermore, we detected *EZH2* expression in 48 paired human BC tissues and the 48 paired normal tissues, and proved that *EZH2* was indeed over-expressed in BC (Figure 3F).

LL22NC03-N64E9.1 Epigenetically Suppresses KLF2 Transcription by Interacting with EZH2

To identify the candidate genes targeting *LL22NC03-N64E9.1* in BC, the expression profile for the genes downstream of *LL22NC03-N64E9.1* in colorectal cancer cells, which was constructed by Lian et al,¹⁷ were reviewed. A qRT-PCR assay was conducted of the *KLF2* as mentioned earlier in the study. The results showed that *LL22NC03-N64E9.1* expression was effectively knocked down, while mRNA levels of *KLF2* were upregulated (Figure 4A). Similarly, *EZH2* knockdown led to the same consistent results (Figure 4B). Moreover, the results of ChIP assays showed that *EZH2* could directly bind to the *KLF2*

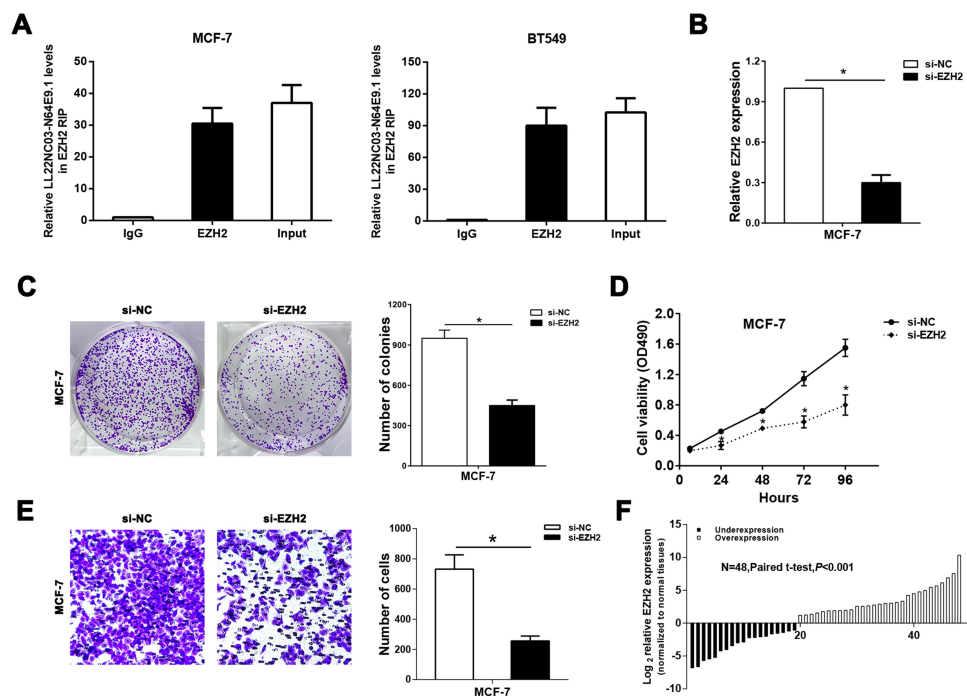


Figure 3 *LL22NC03-N64E9.1* interacts with *EZH2*, which functions as an oncogene in BC. (A) The interactions of *LL22NC03-N64E9.1* with *EZH2* in MCF-7 and BT549 cells were demonstrated using RIP assay. (B) qRT-PCR examined the efficiency of transfecting si-*EZH2* into MCF-7 and BT549 cells. (C and D) Colony formation (C) and MTT (D) assays were conducted to evaluate how the transfection with si-*EZH2* could affect BC cell proliferation. (E) Transwell assays were conducted to assess BC cell migration upon the transfection with si-NC or si-*EZH2*. (F) *EZH2* expression levels in 48 BC tissues and adjacent normal tissues were compared by qRT-PCR. *P<0.05.

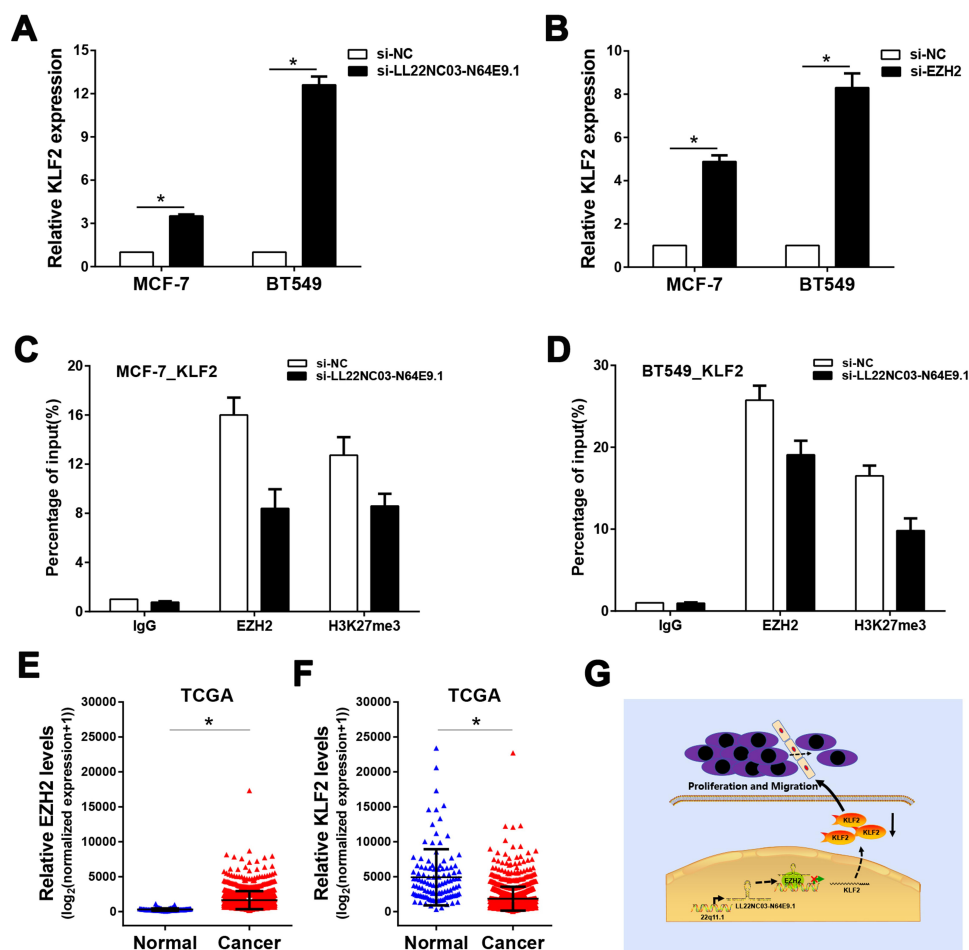


Figure 4 *LL22NC03-N64E9.1* represses *KLF2* transcription via *LL22NC03-N64E9.1/EZH2* pathway. (A) *LL22NC03-N64E9.1* knockdown altered the *KLF2* mRNA expression in MCF-7 and BT-549 cells, as revealed by qRT-PCR. (B) *EZH2* knockdown altered the mRNA expression of *KLF2* in MCF-7 and BT-549 cells, as revealed by qRT-PCR. (C and D) The regulatory effect of *LL22NC03-N64E9.1* on the deposition of *H3K27me3* and the *EZH2* enrichment of the *KLF2* promoter region were validated by ChIP assays. (E) Expression levels of *EZH2* were compared between unpaired human BC tissues and normal tissues, using TCGA data. (F) Expression levels of *KLF2* were compared between unpaired human BC tissues and normal tissues, using TCGA data. (G) A schematic model to illustrate how *LL22NC03-N64E9.1* participated in BC proliferation and migration. * $P < 0.05$.

promoter regions, thereby inducing *H3K27me3* modification in MCF-7 and BT549 cells (Figure 4C and D). Knockdown of *LL22NC03-N64E9.1* led to reduced *EZH2* binding, and the *H3K27me3* occupancy of the *KLF2* promoter locus was also reduced (Figure 4C and D). Additionally, it was found that *EZH2* expression was obviously increased in BC tissues, while *KLF2* expression was lowly expressed in BC tissues (Figure 4E and F). Taken together, *LL22NC03-N64E9.1* promoted BC cell proliferation and migration partly through epigenetically silencing *KLF2* transcription (Figure 4G).

Discussion

LL22NC03-N64E9.1 has attracted considerable interest from researchers, which increased even more due to its recent discovered role in lung and colorectal cancer.^{17,18} For instance,

Jing et al found that *LL22NC03-N64E9.1* was upregulated in lung cancer samples, and it showed a positive association with key clinical parameters of lung cancer, such as overall survival (OS), tumor size, and advanced tumor stage, which could anticipate a poor prognosis.¹⁸ In addition, Lian et al found that *LL22NC03-N64E9.1* was upregulated in colorectal cancer specimens, while its knockdown suppressed malignant cell proliferation.¹⁷ As suggested by previous research, the tumorigenesis and development of BC could be facilitated by abnormally expressed lncRNAs, while other specific lncRNAs have the potential of being candidate diagnostic markers and therapeutic targets against BC. Therefore, proved by this study, *LL22NC03-N64E9.1* expression was remarkably enhanced in BC tissues. Referring to the effects, *LL22NC03-N64E9.1* knockdown suppressed cell proliferation and migration of BC cells via the *LL22NC03-N64E9.1/EZH2* pathway. Moreover,

the lncRNA transcripts could mediate *H3K27* trimethylation in *KLF2* promoters. Thus, *LL22NC03-N64E9.1* could modulate the tumor development in BC, thereby serving as a novel therapeutic target.

To interact with the downstream targets, most lncRNAs directly bind to specific RNA-binding proteins (RBPs). The RNA protein interaction might activate or inactivate downstream gene expression via various mechanisms, including RNA decay, histone protein modification, DNA methylation or chromosome reprogramming.^{23–26} This study demonstrated that *LL22NC03-N64E9.1* could reduce *KLF2* expression in BC cells while *EZH2* was targeted by the lncRNA. As the core subunit within a *PRC2* complex, *EZH2* was the catalyst for the trimethylation of *lysine 27 of histone H3 (H3K27me3)*, which facilitated tumor growth by deactivating the transcription for downstream tumor suppressor genes.²⁷ According to various recent research, dysregulation of *EZH2* is a tumor-related regulatory factor in different types of malignancies.^{28,29} Our study also revealed that *EZH2* was oncogenic in BC cells. *KLF2* is a transcription factor that belongs to the zinc-finger family.^{22,30} Notably, *KLF2* is considered a tumor suppressor gene in a variety of tumors, including BC.^{31,32} Our data showed that *LL22NC03-N64E9.1* could mediate *H3K27* trimethylation in *KLF2* promoters, thus suppressing its expression.

In summary, this study demonstrated that *LL22NC03-N64E9.1* overexpression enhanced BC proliferation and metastasis partially through inhibiting *KLF2* transcription. *LL22NC03-N64E9.1* could be a novel target for antitumor chemotherapy. The identification of the *LL22NC03-N64E9.1/EZH2/KLF2* axis broadened our knowledge and could serve as an important reference in improving BC treatment strategies by considering epigenetic modification. Apart from the achievements, the scope of the study was still limited in some aspects. If the sample size could be increased beyond the current range, the validation of the conclusions would be more creditable, and as follow-up research on the oncogenic functions of *LL22NC03-N64E9.1*, it would be valuable to include in-vivo tests conducted on animal models. In addition, it cannot be excluded that other candidate targets of *LL22NC03-N64E9.1* might also participate in BC development. Thus, follow-up research should be designed to solve the abovementioned shortages, as well as identify the upstream regulatory pathways of *LL22NC03-N64E9.1* overexpression in BC.

Data Sharing Statement

The data that support the findings of this study are available from Weibin Lian upon reasonable request.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed on the journal to which the article will be submitted; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

This study was funded by the Science and Technology Plan Project of Quanzhou (Grant No. 2018N073S) and the Startup Fund for scientific research, Fujian Medical University (Grant No. 2019QH1251).

Disclosure

The authors have declared that no conflict of interest exists.

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