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Long non-coding RNA-CTD-2108O9.1 represses breast cancer metastasis by influencing leukemia inhibitory factor receptor

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Breast cancer (BC) is an aggressive malignant disease in women worldwide with a high tendency to metastasize. However, important biomarkers for BC metastasis remain largely undefined. In the present study, we identified that long non-coding RNA-CTD-2108O9.1 is downregulated in BC tissues and cells and acts as a metastatic inhibitor of BC. Mechanistic investigation determined that lncRNA-CTD-2108O9.1 represses metastasis by targeting leukemia inhibitory factor receptor (LIFR), which is designated as a metastasis suppressor in BC. Our study characterizes a significant tumor suppressor active in BC metastasis repression through the known metastasis inhibitor LIFR.

KEYWORDS
breast cancer, EMT, LIFR, lncRNA, metastasis

1 | INTRODUCTION

Breast cancer (BC) is a global health problem, accounting for the highest cancer incidence rate and high mortality in China and the USA.[1,2] As one of the leading causes of cancer-related death, BC is the most common cancer type in women, and the tumors may progress to become invasive.[1] BC patients who are diagnosed with distant metastasis have distinctly lower survival rates relative to those diagnosed with local and regional stages.[1] Despite the many therapeutic strategies, including radiotherapy, chemotherapy, and surgical excision, metastasis remains refractory and is the ultimate challenge for clinical oncological therapy.[3,4] Thus, finding metastasis-related markers is a pressing need in both clinical and basic research.

Long non-coding RNAs (lncRNAs) are known to affect disease initiation and progression; therefore, in the past, they have been a common target of research. LncRNAs are non-coding transcripts consisting of more than 200 nucleotides, and they are known to affect protein-coding genes by metamorphosis, chromosome rearrangement, repeats, or inserting transposable elements.[5] Despite their variable origins, lncRNAs are ubiquitous and non-nonsense sequences in the genome.[5] LncRNAs have been found to play roles in cardiovascular diseases, neurodegenerative diseases, metabolism-related disorders, bacterium infection, and diverse tumors.[6-9] The aberrant expression of lncRNAs in various cancers has attracted attention, and the biological functions of lncRNAs are increasingly being shown. LncRNA HULC (highly upregulated in liver cancer) was found to be upregulated in hepatocarcinoma, regulating phosphorylation of Y-box binding proteins and increasing translation of some oncogenic mRNAs.[10] DSCAM-AS1 was reported to mediate BC

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development and drug resistance by heterogeneous nuclear ribonucleoprotein L protein.[11] Overexpressed colorectal cancer-associated lncRNA (CCAL) in colorectal cancers was shown to activate Wnt/β-catenin-mediated cancerous progression and indicates poor prognosis.[12] Another novel lncRNA, lncRNA-CTD-2108O9.1, is downregulated in gastric cancer (GC) and its expression level was related to leukemia inhibitory factor receptor (LIFR) in GC.[13] LIFR, the receptor of leukemia inhibitory factor (LIF), has been reported to be a metastatic suppressor in BC.[14] However, the molecular mechanism of how lncRNAs affect LIFR is unclear.

In the present study, we examined lncRNA-CTD-2108O9.1 expression in BC tissues and cell lines and further analyzed the correlation between lncRNA-CTD-2108O9.1 and clinicopathological features of BC. Gain-of-function and loss-of-function experiments were carried out to explore the biological function of lncRNA-CTD-2108O9.1 in BC, identifying its impact on migration, invasion, and epithelial-mesenchymal transition (EMT)-related markers. Furthermore, we proceeded to identify a potential molecular mechanism mediated by lncRNA-CTD-2108O9.1 and its correlation with LIFR in BC tumorigenesis.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human breast cancer cell lines MCF-7 and MDA-MB-231 and human normal breast epithelial cell line MCF-10A were acquired from ATCC (Manassas, VA, USA), and the human breast cancer cell line ZR-75-1 was obtained from the cell bank of Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China). MCF-7 cells were maintained in Eagle's minimum essential medium (EMEM; ATCC) with 0.01 mg/mL insulin (Solarbio, Beijing, China) and 10% FBS, ZR-75-1 cells were cultured in RPMI 1640 medium with 10% FBS, MDA-MB-231 were cultured in L-15 medium with 10% FBS using impermeable flasks, and MCF-10A cells were cultured in DMEM/F12 medium with 5% horse serum, 100 ng/mL cholera, 10 μg/mL insulin, 20 ng/mL epidermal growth factor (EGF) toxin and 500 ng/mL hydrocortisone. All cells were cultured in a humidified incubator at 37°C with 5% CO_2 (Thermo Fisher Scientific, Waltham, MA, USA).

2.2 | Human tissue samples and ethical approval

Ninety-seven BC tissues were obtained from patients who underwent modified radical mastectomy after being diagnosed with BC at the Department of Breast Surgery in the First Affiliated Hospital of China Medical University. Tissues were collected within a half hour following surgery, frozen in liquid nitrogen overnight, and kept at −80°C for long-term storage. All patients signed an ethical consent form.

2.3 | RNA isolation and quantitative real-time PCR

Total RNA of both cells and tissues was extracted and isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA). PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio, Beijing, China) was used for reverse transcription and for producing cDNA. Real-time PCR was carried out in a Light Cycler 480 II Real-Time PCR system (Roche Diagnostics, Basel, Switzerland) using SYBR Green (Takara Bio). GAPDH was used as the internal control.

2.4 | Vectors and transfections

Vectors were transfected to cells with Lipofectamine 2000 Reagent (Invitrogen). According to the product manuals and cellular tolerance, a final concentration of 1.75 μg/mL plasmid or siRNA was used for each transfection in 25 mL impermeable culture flasks (Corning, Corning, NY, USA) with 2 mL culture medium. After 6-hour coculture with vectors, the culture medium was thoroughly exchanged. After lentivirus transfection, stable cells were selected and constructed by puromycin.

2.5 | Migration and invasion assays

Transwell migration assays were carried out with transwells (Corning) and matrigel invasion assays were carried out with matrigel (BD Biosciences, San Jose, CA, USA). Two days after transfection, 2.5×10^5 cells were planted into the upper chamber of the well in 0.2 mL medium with no FBS, and 0.6 mL medium with 10% FBS was added to the lower chamber. After 24 hours, cells were stained with H&E, and the quantity of cells capable of passing through the membrane and matrigel were calculated from 10 random areas across the visual field with a Leica DMI300 microscope (Leica, Wetzlar, Germany).

2.6 | Scratch wound healing assay

Scratch wound healing assays were carried out 24 hours after transfection. Cells were seeded in 6-well plates and incubated for 24 hours. The monolayer was then scratched with a sterile pipette tip and the wound area monitored with a light microscope (DMI3000B; Leica, Bannockburn, IL, USA) at 0, 6, 12, and 24 hours after wounding. Images were analyzed with Image-J software (Media Cybernetics) and ratios were calculated according to the formula (S0 − St)/S0.

2.7 | Cell proliferation assay

Proliferative ability was detected using a CCK-8 (Dojindo Laboratories, Tokyo, Japan) following the manufacturer's instructions. Cells were plated in a 96-well plate at 2500 cells per well and tested at 450 nm wavelength with a microplate reader (Bio-Rad, Hercules, CA, USA).

2.8 | Western blotting analysis

Total proteins were isolated and extracted with a Total Protein Extraction Kit (KeyGen Biotech, Nanjing, China) and Trizol Reagent (Invitrogen). Proteins were separated by SDS-PAGE, transferred to

PVDF membranes (Millipore, Billerica, MA, USA), bound by primary and secondary antibodies, and detected by GelCapture version software (DNR Bio-Imaging Systems, Jerusalem, Israel). β-Actin was used as the internal control.

2.9 | Animal experiments

For in vivo orthotopic xenografts, 30 5-week-old female balb/c nude mice were randomly divided into 5 groups (n = 3). MDA-MB-231 cells (3×10^6) transfected stably in 0.1 mL PBS were injected into right secondary mammary fat pad. Mice were killed on the 35th day after injection and tumors were removed from the body.

For metastasis lung colonization assays, we injected 4×10^6 cells in 0.05 mL PBS and 0.05 mL matrigel into the tail vein of randomly grouped mice (n = 3). On the 53rd day after injection, we carried out 18-fluorodeoxyglucose PET (18F-FDG PET) scans (Metis Micro PET; Madic Technology Co., Ltd, Shandong, China) on mice. 18F-FDG was injected into mice by the tail vein and metabolized for half an hour before the PET scan began.

2.10 | Statistical analyses

All statistical analyses were carried out using SPSS 19.0 software (IBM Corp). Data are listed as mean ± SD and Student's *t* test was used when the variance between groups was similar and Wilcoxon signed rank test was used when the variance between groups was not similar. Expression of lncRNA-CTD-2108O9.1 and patients' clinical characteristics were compared with the χ^2 test.

3 | RESULTS

3.1 | Decreased expression of lncRNA-CTD-2108O9.1 in human BC tissues and cells

We carried out real-time PCR on BC tissue samples and matched adjacent normal tissue samples from 97 patients with BC to quantify expression levels of lncRNA-CTD-2108O9.1. As shown in Figure 1A, lncRNA-CTD-2108O9.1 had lower expression in 88.7% (86 of 97) of BC tissues compared to normal tissues. We further compared lncRNA-CTD-2108O9.1 expression between BC cell lines (MDA-MB-231, MCF-7 and ZR-75-1) and a normal breast epithelial cell line (MCF-10A) and found that lncRNA-CTD-2108O9.1 was downregulated in BC cells compared with normal breast epithelial cells (Figure 1B).

To explore the potential clinical value of lncRNA-CTD-2108O9.1, we analyzed the correlation between its expression and BC clinicopathological characteristics and discovered a relationship between lncRNA-CTD-2108O9.1 and lymph node metastasis. Patients with lower lncRNA-CTD-2108O9.1 were associated with significantly higher lymph node metastasis. We found no other associations between lncRNA-CTD-2108O9.1 and age, tumor size, hormone receptor status or molecular subtypes (Table 1). Collectively, these results suggest that low lncRNA-CTD-2108O9.1 and its correlation with lymph node metastasis in BC patients are of great significance, and that lncRNA-CTD-2108O9.1 may function in metastatic-related processes in BC.

3.2 | Overexpression of lncRNA-CTD-2108O9.1 inhibits BC cell migration and invasion in vitro

To explore whether lncRNA-CTD-2108O9.1 could influence biological functions in BC, we overexpressed lncRNA-CTD-2108O9.1 in MDA-MB-231 and MCF-7 cells, and confirmed the overexpression by real-time PCR (Figure 2A,G). We then carried out transwell migration assays on lncRNA-CTD-2108O9.1-overexpressing cells (pEX2-2108O9.1 cells) and negative control cells (pEX2-NC cells) transfected with lncRNA-CTD-2108O9.1 vectors and empty vectors, respectively. pEX2-2108O9.1 cells had weaker migration compared to pEX2-NC cells (Figure 2B,C,H,I). Invasion, an important component of metastasis, was evaluated by matrigel invasion assay (Figure 2B,D,H,J). The assay indicated that the invasion ability of BC cells was also attenuated after lncRNA-CTD-2108O9.1 overexpression. A scratch assay was then carried out to assess whether lncRNA-CTD-2108O9.1 could impact the migration and motility of BC cells. Differential healing speeds suggested that increasing expression of lncRNA-CTD-2108O9.1 could reduce migration and motility in BC cells (Figure 2E,F,K,L). CCK-8 proliferative assays and cell cycle assays were used to evaluate how lncRNA-CTD-2108O9.1 overexpression affects BC cell proliferation. No significant differences were noted between pEX2-2108O9.1 and pEX2-NC groups in the CCK-8 assay, nor was there any effect on G1, G2, and S phase ratios following lncRNA-CTD-2108O9.1 upregulation (Figure S1).

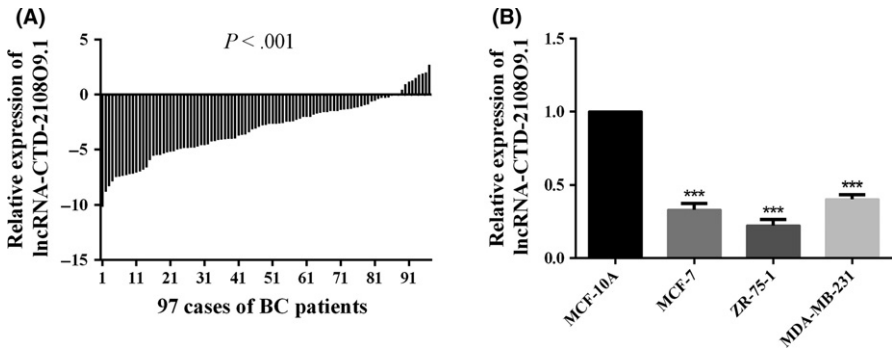

FIGURE 1 Decreased expression of long non-coding RNA (lncRNA)-CTD-2108O9.1 in human breast cancer (BC) tissues and cells. A, Relative expression levels of lncRNA-CTD-2108O9.1 in human BC tissues compared with their matched normal tissues. B, Relative expression in 3 BC cell lines. ***$P < .001$

TABLE 1 LncRNA-CTD-2108O9.1 expression and clinicopathological characteristics in breast cancer patients (n = 97)

	LncRNA-CTD-2108O9.1 expression		
Variable	Low no. (%)	High no. (%)	*P*-value
Age (y)			.087
≤50	13 (26.5)	20 (41.7)	
>50	36 (73.5)	28 (58.3)	
Size (cm)			.584
≤2	9 (18.4)	9 (18.8)	
>2	40 (81.6)	39 (81.3)	
Lymph node		**.023**	
−	24 (49.0)	34 (70.8)	
+	25 (51.0)	14 (29.2)	
ER			.387
−	15 (30.6)	17 (35.4)	
+	34 (69.4)	31 (64.6)	
PR			.089
−	14 (28.6)	21 (43.8)	
+	35 (71.4)	27 (56.3)	
HER2			.272
−	33 (67.3)	36 (75.0)	
+	16 (32.7)	12 (25.0)	
Ki67 (%)			.383
<14	10 (20.4)	12 (25)	
≥14	39 (79.6)	36 (75)	
Molecular subtype			.177
Luminal A	9 (18.4)	9 (18.8)	
Luminal B	18 (36.7)	20 (41.7)	
Luminal B/Her2+	10 (20.4)	2 (4.2)	
Her2+	6 (12.2)	9 (18.8)	
TNBC	6 (12.2)	8 (16.7)	

ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; lncRNA, long non-coding RNA; PR, progesterone receptor; TNBC, triple negative breast cancer.

Bold value indicates *P*-value was lower than .05 with statistical significance.

3.3 | Knockdown of lncRNA-CTD-2108O9.1 increases BC cells migration and invasion in vitro

To complement the above experiments, we next knocked down lncRNA-CTD-2108O9.1 through siRNA. MDA-MB-231 cells and MCF-7 cells were transfected with lncRNA-CTD-2108O9.1-silenced vectors cells (si-2108O9.1 cells) or scrambled vectors (si-NC cells). We repeated the above metastasis and proliferation studies on these cells. The transwell migration and matrigel invasion assays showed that silencing lncRNA-CTD-2108O9.1 could significantly increase BC cell migration and invasion (Figure 3A-C,F-H). Scratch assay confirmed that BC cell migration and motility were intensified following lncRNA-CTD-2108O9.1 downregulation (Figure 3D,E,I,J). Similar to before, the above analysis suggested that lncRNA-CTD-2108O9.1 can regulate the migratory and invasive capabilities of BC cells.

3.4 | Matrix metalloproteinase and EMT-related markers are involved in lncRNA-CTD-2108O9.1-induced metastatic decrease

To assess the potential mechanism underlying the effect of lncRNA-CTD-2108O9.1 on BC metastasis, we examined MMP family members and EMT markers by real-time PCR and western blotting. As shown in Figure 4, real-time PCR showed that invasion-related markers MMP-9 and MMP-2 and mesenchymal markers N-cadherin, vimentin, β-catenin, and α-smooth muscle actin (α-SMA) were both downregulated, whereas epithelial markers laminin and ZO-1 were both upregulated in pEX2-2108O9.1-overexpressing cells (Figure 4A, B). Western blotting showed consistent results, with increased laminin and ZO-1 and decreased MMP-2, MMP-9, N-cadherin, vimentin, and β-catenin (Figure 4C,D). We took phase-contrast micrographs of lncRNA-CTD-2108O9.1-overexpressing cells and the control group and found that overexpression of lncRNA-CTD-2108O9.1 enhanced morphological features of EMT (Figure 4E). Immunofluorescence staining assays were done and we observed that MMP-9 and β-catenin decreased whereas cytokeratin increased following lncRNA-CTD-2108O9.1 overexpression (Figure 4F). These findings showed that EMT-related markers and MMP may be involved in the mechanism of lncRNA-CTD-2108O9.1 regulating migration and invasion.

To evaluate the relationship between expression levels of lncRNA-CTD-2108O9.1 and MMP family and EMT-related markers, we detected MMP family and EMT-related markers in BC tissues by real-time PCR and the expression level of each marker is shown in Figure S2A. We then analyzed the correlation between them and found that laminin ($r = .488$, $P < .01$) and β-catenin ($r = .334$, $P < .01$) were moderately correlated to lncRNA-CTD-2108O9.1 in BC tissues, whereas snail ($r = .282$, $P < .05$), N-cadherin ($r = .261$, $P < .05$) and twist ($r = .244$, $P < .05$) were weakly related with lncRNA-CTD-2108O9.1 (Table 2).

3.5 | Coordinated expression of lncRNA-CTD-2108O9.1 and LIFR in BC tissues and cells

We examined the expression of LIFR relative to lncRNA-CTD-2108O9.1 by real-time PCR and found that LIFR transcript levels were downregulated in 97 cases of BC patient tissue samples (Figure 5A). Thus, correlation between lncRNA-CTD-2108O9.1 and LIFR RNA expression levels was analyzed. We found that correlation coefficient was 0.393 ($P < .01$) (Figure S2C), suggesting a moderate relevance between lncRNA-CTD-2108O9.1 and LIFR RNA expression levels, although their overall expression trends were similar. Interestingly, we randomly selected several pairs of BC tissues and tested LIFR expression level in tissues by western blot and found LIFR protein expression level was positively related to lncRNA-CTD-2108O9.1 RNA expression level in the same BC tissue samples (Figure S2B). To further investigate the relationship between lncRNA-CTD-2108O9.1 and LIFR, we measured expression of LIFR in pEX2-2108O9.1 and si-2108O9.1 cells and their respective scrambled controls at both the transcriptional and translational levels. Conversely, real-time PCR showed no significant differences

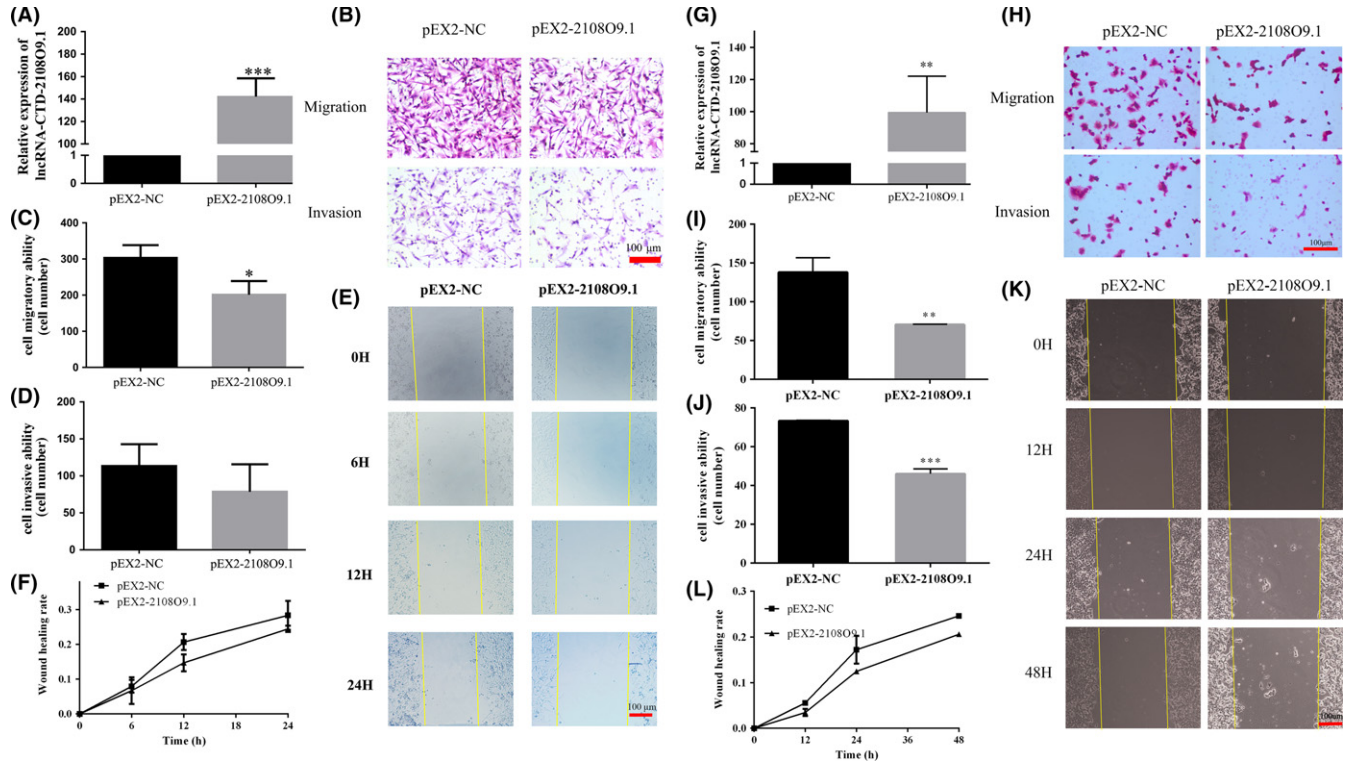

FIGURE 2 Overexpression of long non-coding RNA (lncRNA)-CTD-2108O9.1 inhibits breast cancer (BC) cell migration and invasion in vitro. A, Transfection efficacy of pEX2-2108O9.1 was detected 48 h after transfection in MDA-MB-231 cells. B-D, In MDA-MB-231 cells, transwell assays were carried out to evaluate the involvement of lncRNA-CTD-2108O9.1 in BC metastasis. E,F, In MDA-MB-231 cells, scratch wound healing assays identified the role of lncRNA-CTD-2108O9.1 on the migration ability of BC cells. G, Transfection efficacy of pEX2-2108O9.1 was detected 48 h after transfection in MCF-7 cells. H-J, In MCF-7 cells, transwell assays were carried out. K,L, In MCF-7 cells, scratch wound healing assays were carried out. *$P < .05$, **$P < .01$, ***$P < .001$

in LIFR transcriptional levels between lncRNA-CTD-2108O9.1-overexpressing cells and their negative controls (Figure 5B,C), yet western blotting indicated that LIFR protein levels were raised upon lncRNA-CTD-2108O9.1 overexpression and reduced when lncRNA-CTD-2108O9.1 was knocked down (Figure 5D,E). We detected expression level of eukaryotic translation initiation factor 4 gamma 1 (EIF4G1) by western blotting and found a relevant increase of EIF4G1 in pEX2-2108O9.1 cells (Figure 5F). Thus, we pulled down the proteins that can directly bind to lncRNA-CTD-2108O9.1 and detected EIF4G1 expression level by western blot. Results suggested that lncRNA-CTD-2108O9.1 can directly target EIF4G1 (Figure 5G), indicating that lncRNA-CTD-2108O9.1 may be an upstream regulator of LIFR expression at the translational level.

3.6 | Knockdown of LIFR restores impaired migration and invasion ability induced by lncRNA-CTD-2108O9.1

Previous research has shown that LIFR can suppress BC metastasis; therefore, we studied whether lncRNA-CTD-2108O9.1 functioned through LIFR to elicit its repression of BC cell migration and invasion. We knocked down LIFR in a background of lncRNA-CTD-2108O9.1 overexpression. Transwell migration and matrigel invasion assays showed that migration and invasion suppressed by lncRNA-CTD-2108O9.1 overexpression were restored in BC cells cotransfected with lncRNA-CTD-2108O9.1 and sh-LIFR compared with cells cotransfected with lncRNA-CTD-2108O9.1 and scrambled sh-NC (Figure 6A-C,F-H). Scratch assays supported this result, illustrating that the inhibition of metastasis resulting from overexpressed lncRNA-CTD-2108O9.1 could be abolished when LIFR was removed (Figure 6D,E,I,J). Taken together, these results imply that lncRNA-CTD-2108O9.1 can target LIFR and affects BC metastasis through a LIFR-dependent pathway.

To further explore the mechanism underlying the impact of lncRNA-CTD-2108O9.1 on BC, MS2-tagged RNA affinity purification (TRAP) was used to isolate RNA molecules that associated with lncRNA-CTD-2108O9.1 directly. Consequently, we carried out real-time PCR on the isolated molecules and identified that LIFR, MMP-2, MMP-9, laminin, N-cadherin and β-catenin were enriched to combine with lncRNA-CTD-2108O9.1 directly (Figure 6K). This result may explain the correlation of the above markers and lncRNA-CTD-2108O9.1 in tissues and cells. It was strongly suggested that the following well-known metastasis-related markers including LIFR, MMP-2, MMP-9, laminin, N-cadherin and β-catenin may be directly targeted and regulated at post-transcriptional level by lncRNA-CTD-2108O9.1.

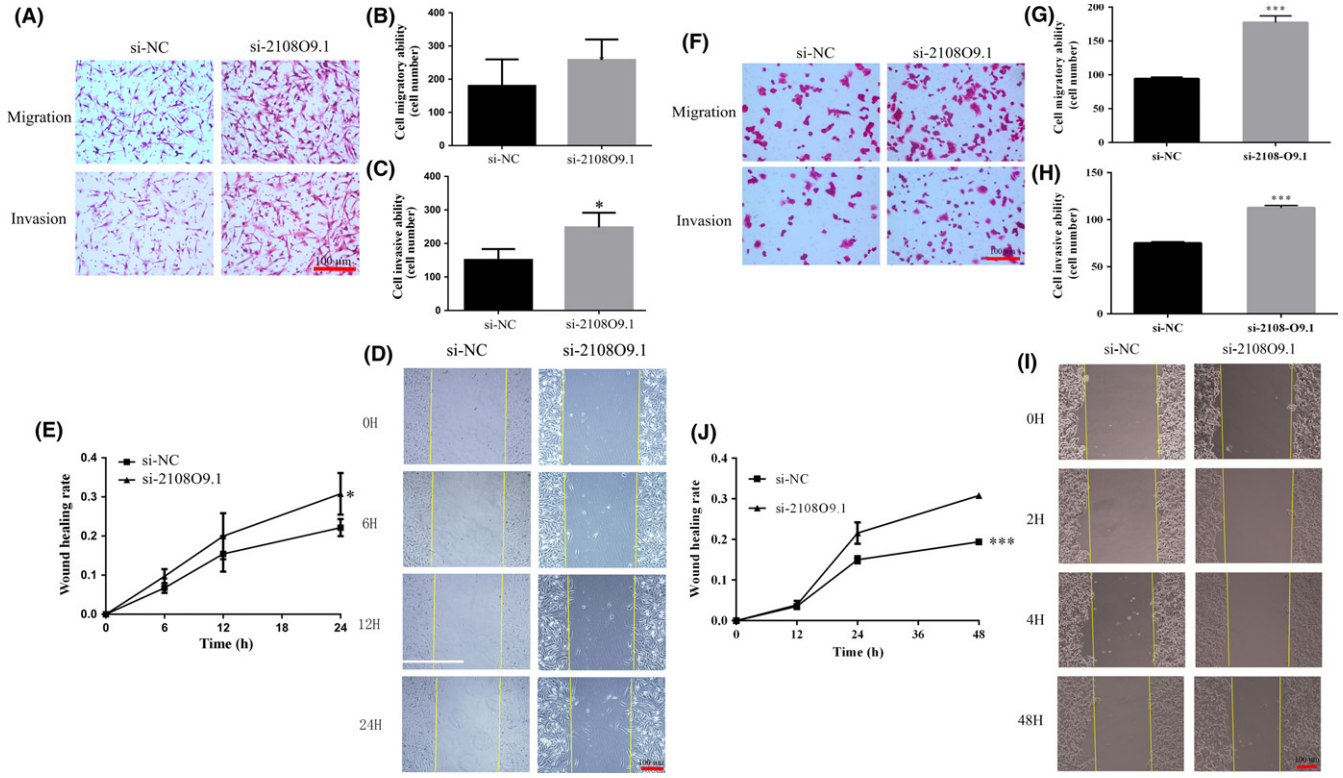

FIGURE 3 Knockdown of long non-coding RNA (lncRNA)-CTD-2108O9.1 increases breast cancer (BC) cell migration and invasion in vitro. A-C, Transwell assays were carried out to evaluate the involvement of lncRNA-CTD-2108O9.1 in BC metastasis in MDA-MB-231 cells. D,E, In MDA-MB-231 cells, scratch wound healing assays identified the role of lncRNA-CTD-2108O9.1 on the migration ability of BC cells. F-H, Transwell assays were carried out to evaluate the involvement of lncRNA-CTD-2108O9.1 in BC metastasis in MCF-7 cells. I, J, In MCF-7 cells, scratch wound healing assays were carried out. $*P < .05$, $***P < .001$

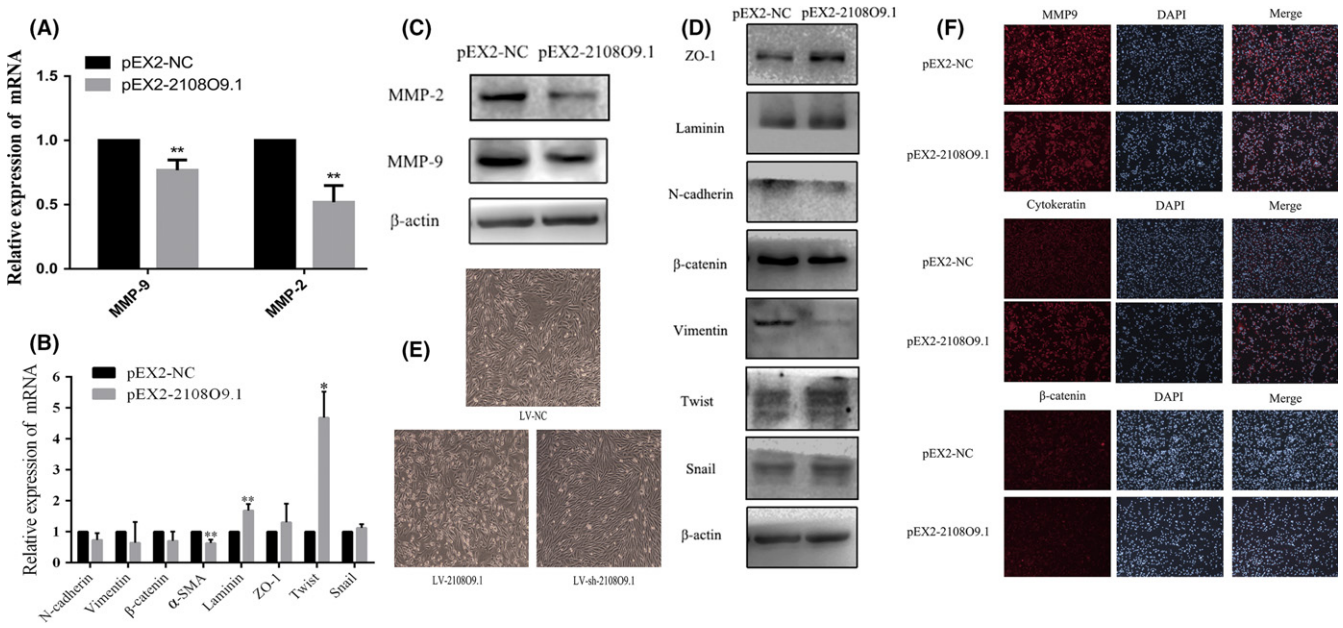

FIGURE 4 MMP and epithelial-mesenchymal transition (EMT)-related markers are involved in long non-coding RNA (lncRNA)-CTD-2108O9.1-induced decline of metastasis. A, Transcriptional expression levels of MMP-9 and MMP-2 were detected with real-time PCR. B, Transcriptional expression levels of N-cadherin, vimentin, β-catenin, α-smooth muscle actin (α-SMA), laminin, and ZO-1 were detected with real-time PCR. C, Translational expression levels of MMP-9 and MMP-2 were detected with western blot. D, Translational expression levels of ZO-1, laminin, N-cadherin, β-catenin, and vimentin were detected with western blot. $**P < .01$. E, Phase-contrast micrographs of transfected MDA-MB-231 cells. F, Immunofluorescence staining of MMP-9, β-catenin and cytokeratin

3.7 | LncRNA-CTD-2108O9.1 suppresses BC metastasis through LIFR in vivo

To evaluate the metastasis inhibition ability of lncRNA-CTD-2108O9.1 in vivo, we carried out both mice orthotopic xenografts and lung colonization assays in nude mice. Stable MDA-MB-231 cells were constructed for various groups, including LV-NC, LV-2108O9.1, LV-sh-2108O9.1, LV-2108O9.1 + shLIFR and LV-sh-2108O9.1 + LIFR. BC cells in these groups were injected into the tail vein of nude mice for lung colonization assays. We carried out PET scanning on nude mice and discovered that maximum standardized uptake value (SUVmax) of the LV-2108O9.1 group (0.49 ± 0.07) was lower and that of the SUVmax LV-sh-2108O9.1 group (1.20 ± 0.19) was significantly higher compared to that of the LV-NC group (0.70 ± 0.10). Moreover, SUVmax of the LV-2108O9.1 + shLIFR group (0.59 ± 0.04) increased more than that of the LV-2108O9.1 group, whereas that of the LV-sh-2108O9.1 + LIFR group (0.85 ± 0.02) decreased more than that of the LV-sh-2108O9.1 group (Figure 7A,B).

Additionally, the same groups of cells were injected into the mammary fat pad of nude mice to construct the orthotopic xenografts model. However, tumor growth, weight and diameter did not show any significant difference among these groups (Figure S3). Experiments above indicated that lncRNA-CTD-2108O9.1 could suppress BC metastasis through LIFR in vivo, but lncRNA-CTD-2108O9.1 had no significant influence on tumor proliferation, recapitulating the results of cell function experiments in vitro.

TABLE 2 Correlation of lncRNA-CTD-2108O9.1 and metastasis-related markers

	lncRNA-CTD-2108O9.1		
	r-value	*P*-value	n
MMP-2	−.008	.936	95
MMP-9	.153	.138	95
Laminin	.488**	<.01	82
β-catenin	.334**	.001	95
N-cadherin	.261*	.024	75
Twist	.244*	.017	95
Snail	.282*	.011	80
Vimentin	−.152	.151	91
ZO-1	.063	.542	96

lncRNA, long non-coding RNA.
*$P < .05$, **$P < .01$.

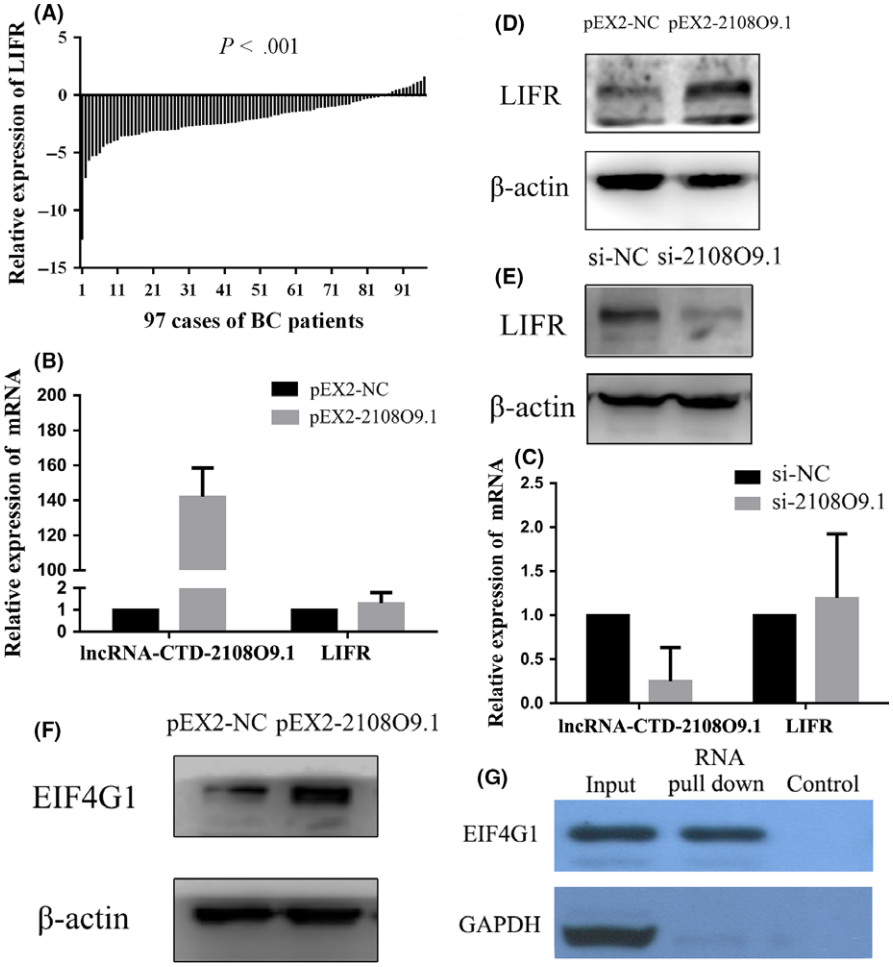

FIGURE 5 Coordinated expression of long non-coding RNA (lncRNA)-CTD-2108O9.1 and leukemia inhibitory factor receptor (LIFR) in breast cancer (BC) tissues and cells. A, Relative expression levels of LIFR in human BC tissues compared with their matched normal tissues. B, Expression of LIFR was detected by real-time PCR after lncRNA-CTD-2108O9.1 overexpression. C, Expression of LIFR was detected by real-time PCR after lncRNA-CTD-2108O9.1 was silenced. D, Expression of LIFR was detected by western blot after lncRNA-CTD-2108O9.1 overexpression. E, Expression of LIFR was detected by western blotting after lncRNA-CTD-2108O9.1 was knocked down. F, Expression of EIF4G1 was detected by western blotting after lncRNA-CTD-2108O9.1 overexpression. G, Western blot after RNA pull-down

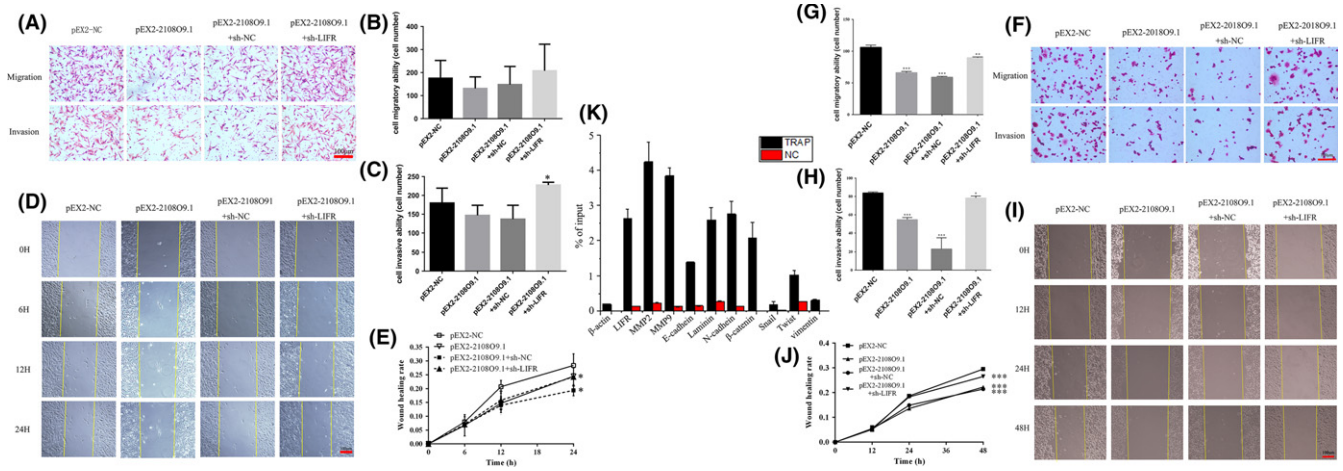

FIGURE 6 Knockdown of leukemia inhibitory factor receptor (LIFR) restores the impaired migration and invasion ability induced by long non-coding RNA (lncRNA)-CTD-2108O9.1. A-C, Transwell assays were carried out to identify whether lncRNA-CTD-2108O9.1 suppresses breast cancer (BC) metastasis through LIFR in MDA-MB-231 cells. D,E, Scratch wound healing assays were carried out to validate whether lncRNA-CTD-2108O9.1-induced repression of the migration ability of BC cells could be restored by LIFR in MDA-MB-231 cells. F-H, Transwell assays were carried out in MCF-7 cells. I,J, Scratch wound healing assays were carried out In MCF-7 cells. K, Real-time PCR was carried out after MS2-TRAP. $*P < .05$, $**P < .01$, $***P < .001$

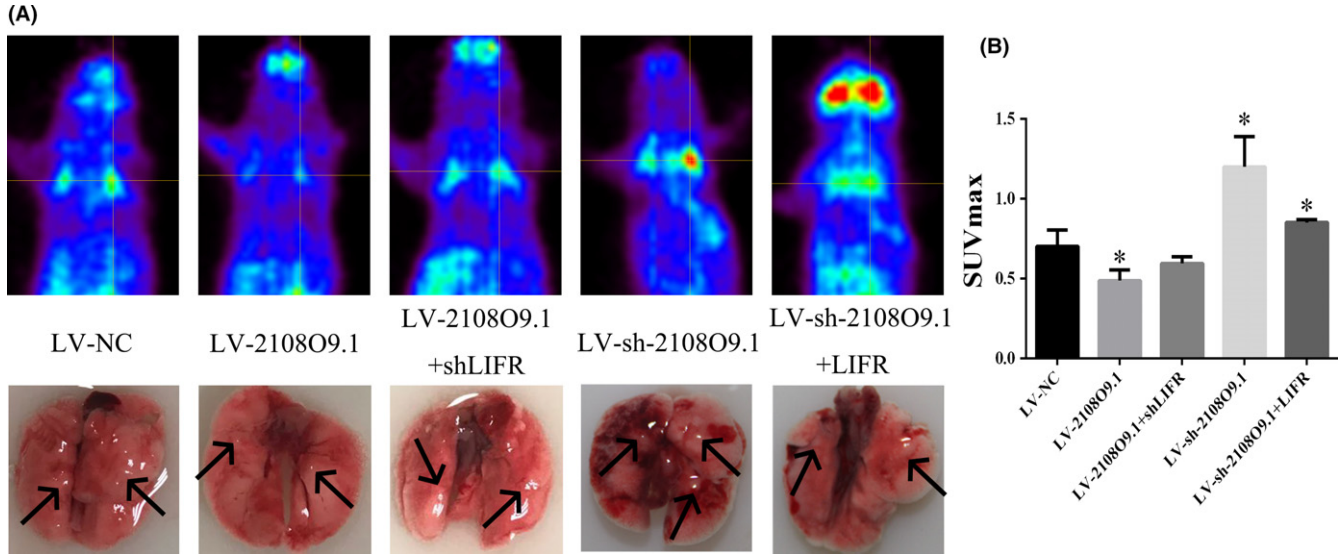

FIGURE 7 Long non-coding RNA (lncRNA)-CTD-2108O9.1 suppresses breast cancer metastasis through leukemia inhibitory factor receptor (LIFR) in vivo. A, B, Transverse section of 18F-FDG PET images of mice on the 53rd day after tail vein injection with 3×10^6 MDA-MB-231 cells and the max SUV were analyzed in each group, arrows point at metastatic lesion, n = 3, $*P < .05$

4 | DISCUSSION

Breast cancer has been the leading cause of cancer-related deaths in past decades.[1,15] A paucity of techniques for early detection results in a high ratio of BC patients being diagnosed at advanced stages with metastasis already present. Intrinsic and extrinsic regulators and mechanisms of BC metastasis have been studied ardently yet remain unclear. Although lncRNAs do not code for proteins directly, they are seen to be aberrantly expressed in many diseases.[16-19] Studies in many malignant tumors show that lncRNA dysregulation may associate with clinical features. For example, highly expressed lncRNA SChLA (*S*econd *Ch*romosome *L*ocus *A*ssociated with *P*rostate-1) was shown to forecast poorer outcomes in prostate cancers,[17] and lncRNA PRAL (p53 regulation-associated lncRNA) is regarded as a cancer promotor and associates with decreased survival in hepatocellular cancer.[18] In the present study, we detected the expression of lncRNA-CTD-2108O9.1 in BC tissues and cell lines and surprisingly found lncRNA-CTD-2108O9.1 to be downregulated in BC tissues and BC cells compared to paired normal tissues and healthy epithelial cells. To determine the significance of lncRNA-CTD-2108O9.1 in BC, we analyzed the relationship between lncRNA-CTD-2108O9.1 expression and corresponding clinical pathological

parameters, noting a correlation between lncRNA-CTD-2108O9.1 expression and lymph node metastasis. This indicated that lncRNA-CTD-2108O9.1 is involved in BC progression and may provide clues for potential future clinical applications.

In addition to their dysregulated expression and clinical associations, the involvement of lncRNAs in cancer initiation, progression, and prognosis has been eagerly studied.[20-22] Examples include lncRNA MRCCAT1 (metastatic renal cell carcinoma-associated transcript 1) expression being raised in metastatic foci with the ability to strengthen metastasis in clear cell renal cell carcinoma[23] and the high expression of lncRNA CRNDE (colorectal neoplasia differentially expressed) associated with enlarged tumor size, advanced pathological stages, and reinforcing apoptotic and proliferative capacity in colorectal cancer.[24] In the present study, we overexpressed and silenced lncRNA-CTD-2108O9.1 to examine its biological function in BC cells. We observed that BC cell migration and invasion were repressed by increased lncRNA-CTD-2108O9.1 and intensified with deficient lncRNA-CTD-2108O9.1, yet proliferation was not obviously influenced. These results intimate that lncRNA-CTD-2108O9.1 might inhibit the metastatic ability of BC, serving as a metastasis suppressive gene.

The regulation patterns and mechanisms by which lncRNAs act on cancer are numerous and complicated. Some lncRNAs occupy binding sites of RNAs and proteins, serving as decoys.[25] Some associate with protein factors to activate transcription and chromatin regulation, acting as enhancers.[26] Others act as scaffolds to modulate interactions between proteins and genes, thus affecting transcription of oncogenes or anti-oncogenes.[27] Antisense lncRNA (aslncRNA) are a special type of lncRNA reported to interfere with proximal genes through cis-acting effects to regulate transcription and post-transcriptional modifications.[28,29] LncRNA-CTD-2108O9.1, also called EGFLAM-AS1 and located in the chromosome 5p13.1 region, is the antisense of the EGFLAM gene. However, studies on EGFLAM mainly show its function on visual perception, seldom on the oncological procedure.[30] We were attracted to another gene, LIFR, which is also in the chromosome 5p13.1 region, within 50 kbp downstream of lncRNA-CTD-2108O9.1. LIFR is the receptor of LIF, which serves as a cytokine complex with oncostatin M.[31] Interestingly, LIFR is downregulated and regarded as a tumor suppressor gene in gastric cancers, hepatocellular carcinoma, breast cancer, pancreatic cancers and colorectal carcinoma.[13,14,32-34] Chen et al[14] identified LIFR as a metastatic suppressor by activating the Hippo kinase cascade and inactivating YES-associated protein (YAP) in BC. Additionally, Johnson et al[35] suggested that LIFR expression alleviates bone metastasis through the LIFR-STAT3 pathway and is negatively related to good prognosis. We analyzed the expression correlation between lncRNA-CTD-2108O9.1 and LIFR in BC tissues and cells, finding a moderate correlation coefficient between them. At the same time, we examined LIFR expression at both the mRNA and protein levels upon lncRNA-CTD-2108O9.1 overexpression or silencing in BC cells, observing that LIFR protein expression positively related to lncRNA-CTD-2108O9.1 but seeing no change at the mRNA level. To explain this, we predicted a potential interaction between EIF4G1 and lncRNA-CTD-2108O9.1, and subsequently observed increased EIF4G1 with overexpressed lncRNA-CTD-2108O9.1, implicating EIF4G1 in the deficiency of lncRNA-CTD-2108O9.1. RNA pull-down and western blot experiments proved that EIF4G1 can directly bind to lncRNA-CTD-2108O9.1. These phenomena may indicate a possible mechanism of how lncRNA-CTD-2108O9.1 could regulate LIFR expression at the translational level. To further prove the association of lncRNA-CTD-2108O9.1 and LIFR, we knocked down LIFR in BC cells expressing exogenous lncRNA-CTD-2108O9.1 and found that LIFR can restore the impaired metastasis resulting from increased lncRNA-CTD-2108O9.1. Based on MS2-TRAP experiment and bioinformatics studies, we found that lncRNA-CTD-2108O9.1 could inhibit BC metastasis through direct binding to known metastasis-related markers, including LIFR, MMP-2, MMP-9, laminin, N-cadherin and β-catenin.

In conclusion, to the best of our knowledge, our study is the first to explore which transcript mediates LIFR and inhibits metastasis at the lncRNA level (Figure 8). We validated the reduced expression of lncRNA-CTD-2108O9.1 in BC and its inhibitory effects on metastasis both in vitro and in vivo. We further researched possible molecular mechanisms of lncRNA-CTD-2108O9.1 through EMT-related genes and its association with the metastatic inhibitor LIFR, and proved that lncRNA-CTD-2108O9.1 could directly target LIFR, MMP-2, MMP-9, laminin, N-cadherin and β-catenin. Nevertheless, the detailed mechanism of the interaction between lncRNA-CTD-2108O9.1 and LIFR remains unclear. Although lymph node metastasis significantly correlates with lncRNA-CTD-2108O9.1 expression, data on prognosis and overall survival are absent as a result of the short time span since sample collection and the relatively good

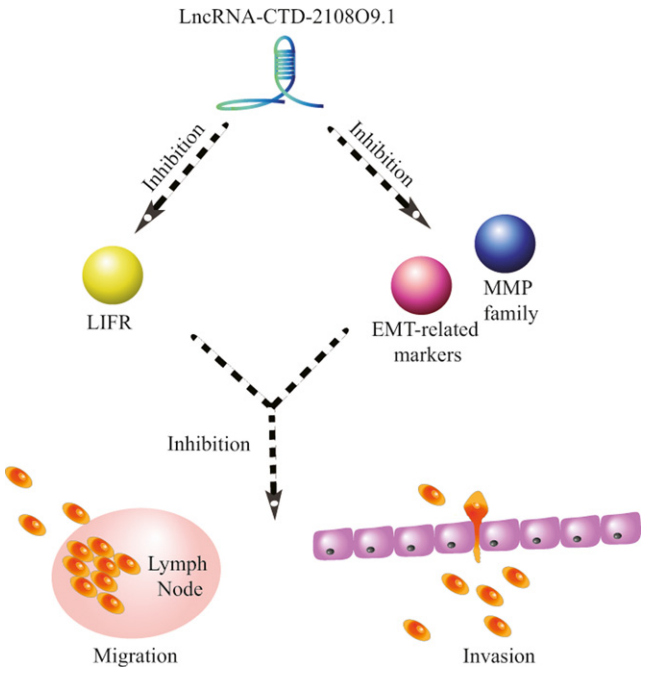

FIGURE 8 Downregulation of long non-coding RNA (lncRNA)-CTD-2108O9.1 in breast cancer and its inhibitory effects on metastasis through its associated metastatic inhibitor leukemia inhibitory factor receptor (LIFR), epithelial-mesenchymal transition (EMT)-related genes and MMP family

prognosis of BC. Accordingly, patient follow ups will be monitored and analyzed, and we will further investigate the mechanism of how lncRNA-CTD-2108O9.1 regulates LIFR. However, our current research is sufficient to indicate that lncRNA-CTD-2108O9.1 can significantly suppress BC metastasis and that it may be a potential biomarker and therapeutic target for BC.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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