**ARTICLE**

**LINC00589-dominated ceRNA networks regulate multiple chemoresistance and cancer stem cell-like properties in HER2+ breast cancer**

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**INTRODUCTION**

Breast cancer is the most prevalent malignancy, with high worldwide mortality in women. Overexpression of human epidermal growth factor receptor 2 (HER2) occurs in 25–30% of breast cancers and is associated with poor prognosis1,2. Though HER2-targeted therapy, such as trastuzumab, improves survival dramatically and is the most highly recommended treatment for HER2-positive patients with early-stage or metastatic breast cancer, high rates of inherent or acquired trastuzumab resistance pose a major obstacle3,4. In addition, emerging evidence suggest that trastuzumab resistance is closely associated with epithelial–mesenchymal transition (EMT), multiple drug resistance (MDR), and cancer stem cell (CSC)-like properties, which make it more complex to treat trastuzumab-resistant breast cancers5. However, key regulatory nodes that are concurrently involved in trastuzumab resistance, MDR, and CSC properties have yet to be uncovered. It is urgent to identify molecular links that concurrently regulate these processes as an approach to developing more effective therapeutic targets.

Noncoding RNAs (ncRNAs) compose the large majority (~98%) of human transcriptome and participate as key players in diverse biological processes6. Long noncoding RNAs (lncRNAs) are a class of ncRNAs that are longer than 200 nucleotides and have limited or no protein-coding capacity7. LncRNAs, in conjunction with microRNAs or other signaling partners, regulate complex networks, and recent evidence is emerging for key roles of lncRNAs as regulators and potential targets in breast cancer. For example, LncRNA CARMN, acting via the miR143-3p host gene, counteracts cisplatin resistance in triple-negative breast cancer and is associated with positive prognosis8. LncRNA CCAT1 interacts with miR-204/211, miR-148a/152, and Annexin A2, and consequently promotes breast cancer stem cell function by activating WNT/β-catenin signaling9. Our previous reports also suggest that lncRNAs, including LncRNA UCA1, miR-200c, miR-221, and miR-375, reverse trastuzumab resistance of HER2-positive breast cancer10,11. Therefore, extensive investigation of lncRNAs is important for the development of novel diagnostic and therapeutic targets in breast cancer.

Long intergenic non-protein-coding RNA 589 (LINC00589, NCBI gene ID: 619351), also known as TSLNC8, is located on Chromosome 8p12 and has been validated as a non-protein-coding RNA. In hepatocellular carcinoma, non-small cell lung cancer, and glioma, LINC00589 inhibits proliferation, invasion, and metastasis12. On the other hand, in pancreatic cancer, LINC00589 serves as an oncogene by stabilizing CTNNB113. These results imply that LINC00589 is important in cancer progression but that its functions vary among different cancer types14,15. Nevertheless, the role of LINC00589 in breast cancer has not been elucidated.

In this study, we evaluate the expression of LINC00589 in trastuzumab-resistant breast cancer tissues and cell lines, and analyze its association with patient prognosis. We also perform gain- and loss-of-function experiments to explore the biological roles of LINC00589 in trastuzumab resistance, MDR, and CSC properties in vitro and in vivo. Furthermore, we investigate the molecular mechanisms whereby LINC00589 exerts its diverse functions in HER2 breast cancer. Our data show that LINC00589...
concurrently modulates trastuzumab resistance, MDR and CSC-like properties of HER2-positive breast cancer. Further mechanistic investigations reveal that LINC00589 serves as a competing endogenous RNA (ceRNA) to regulate DLG5 and PRDM16 expression through binding miR-100 and miR-452. Thus, LINC00589 is a key node for simultaneously controlling trastuzumab resistance, MDR, and CSC-like properties in breast cancer with potential therapeutic value.

RESULTS
LINC00589 expression is decreased in trastuzumab-resistant breast cancer and is correlated with the prognosis of HER2-positive breast cancer
The long noncoding RNA LINC00589, is located on Chromosome 8p12 and contains four exons (Supplementary Fig. 1a). Its full-length 1413 bp nucleotides (Supplementary Fig. 1b) and the secondary structure (Supplementary Fig. 1c) were shown. Although LINC00589 has been reported to suppress cell proliferation in hepatocellular carcinoma and non-small cell lung cancer, its biological roles are largely unknown. Especially, its roles in drug resistance to breast cancer have not been investigated prior to this study. Therefore, to uncover the potential functions of LINC00589 in trastuzumab-resistant breast cancer, we obtained biopsies from 71 HER2-positive breast cancer patients who received trastuzumab treatment. Based on the immuno-related response evaluation criteria in solid tumors, the patients were divided into two groups: the responding group (CR + PR, 38 cases) and the non-responding group (SD + PD, 33 cases). qRT-PCR analysis of biopsies revealed a dramatically lower expression of LINC00589 in the trastuzumab non-responding group than in the responding group (Fig. 1A). To investigate the potential predictive value of LINC00589 expression, we established a ROC curve to differentiate the responding patients from the non-responding patients. The area under the curve (AUC), diagnostic sensitivity, and specificity reached 0.808, 78.8% and 80.0%, respectively, with the established cut-offs (2.785) (Fig. 1B). For further verification, we divided the samples into high or low LINC00589 expression groups according to the cutoff value, and the proportion of responding patients was significantly higher in the high LINC00589 expression group (81.08%) than in the low LINC00589 expression group (23.53%) (Fig. 1C). These results suggest that LINC00589 may serve as a diagnostic marker for trastuzumab-responding patients.

To further evaluate the prognostic value of LINC00589 expression, we obtained formalin-fixed and paraffin-embedded (FFPE) samples from an independent cohort of 92 trastuzumab-treated HER2-positive breast cancer patients with available clinical data. High and low expression of LINC00589 were determined by ISH as represented in Fig. 1D. The results suggest that there is no obvious correlation between LINC00589 expression and age, menopausal status, histologic grade, lymph node status, ER status or PR status in HER2-positive breast cancer patient tissues; however, LINC00589 expression was significantly correlated with TNM stage (Table 1). Furthermore, Kaplan–Meier analysis indicated that LINC00589 expression in trastuzumab-resistant breast cancer patients with high LINC00589 expression had a better overall survival than those with low LINC00589 expression (Fig. 1E). In addition, multivariate Cox regression analysis revealed that LINC00589 expression and lymph node status provided independent prognostic factors for overall survival in the HER2-positive breast cancer patients (Table 2). To provide additional support for the correlation of LINC00589 expression with trastuzumab resistance, we treated SKBR3 breast cancer cells with 5 μg/mL trastuzumab for 6 months, as previously described and obtained 6 trastuzumab-resistant (TR) clones. The IC50 for 6 TR clones was much higher than WT clone, and IC50 value of 6# TR clones was 24 μg/mL (Supplementary Fig. 2). LINC00589 expression was dramatically lower in all the TR cell clones than in the wild-type cells (WT) (Fig. 1F). The 6# TR cell clone that expresses the lowest LINC00589 was selected for further investigation the role of LINC00589 in trastuzumab resistance. Compared with WT cells, the TR cells showed more resistant to trastuzumab treatment, as evidenced by elevated cell viability and IC50 (Supplementary Fig. 3). Altogether, our data indicate that LINC00589 expression is downregulated in trastuzumab-resistant breast cancer and correlates with patient survival, suggesting that LINC00589 may be a valuable diagnostic marker for discriminating trastuzumab responders and a prognostic marker for predicting the survival of HER2-positive breast cancer patients.

LINC00589 counteracts trastuzumab resistance in HER2-positive breast cancer
To determine the functional role of LINC00589 in trastuzumab resistance of HER2-positive breast cancer cells, we constructed lentiviruses that overexpress or silence LINC00589 (Supplementary Fig. 4a, b). WT and TR SKBR3 and HER2-overexpressing BT474 cells were infected with Lv-NC or Lv-LINC00589 IncRNA expression vector, or sh-NC or sh-LINC00589 lentivirus. CCK-8 assays revealed that overexpression of LINC00589 decreased the cell viability of all the six TR SKBR3 cells (Fig. 2A and Supplementary Fig. 5), while knockdown of LINC00589 increased cell viability in WT SKBR3 and BT474 breast cancer cells, which was verified under increasing doses (Supplementary Fig. 6a–c) or times (Fig. 2B, C) of trastuzumab treatment. In addition, the apoptosis rate of TR cells was increased by LINC00589 overexpression after trastuzumab treatment, while the apoptosis rate of WT cells was decreased by LINC00589 silencing (Fig. 2D, E). We also investigated whether LINC00589 regulates the anchorage-independent growth of HER2-positive breast cancer cells. The data showed that LINC00589 upregulation suppressed the number of soft agar colonies formed in TR cells, while knockdown of LINC00589 increased the number of soft agar colonies formed in WT cells (Fig. 2F, G). Collectively, these findings indicate that LINC00589 re-sensitizes resistant breast cancer cells to trastuzumab.

LINC00589 reverses cancer stem cell-like properties and multiple chemoresistance in trastuzumab-resistant HER2-positive breast cancer
Based on increasing evidence that trastuzumab-resistant breast cancer cells exhibit CSC-like properties, we sought to determine whether LINC00589 is associated with stemness and multiple chemoresistance in breast cancer cells. The ability to form mammospheres in ultra-low–attaching culture conditions is a common characteristic of CSC-like cells. As shown in Fig. 3, the average number and volumes of the spheres derived from the LINC00589-overexpressed trastuzumab-resistant cells were lower than those derived from control cells (Fig. 3A–C). We also examined the expression status of CD24, CD44, CD133, Nanog, OCT4, and SOX2, which have been extensively used as molecular markers for breast CSCs. When LINC00589 was overexpressed in TR cells, CD24 (a negative marker of CSC) was upregulated, and CD44, CD133, Nanog, OCT4, and SOX2 (positive markers of CSC) were downregulated, which was demonstrated at both the mRNA and protein levels (Fig. 3D, E). These results indicated that LINC00589 was an important regulator of CSC-like properties in breast cancer.

Given that CSC-like properties are thought to constitute a leading cause for multiple drug resistance of various cancers, we hypothesized that trastuzumab-resistant breast cancer cells might acquire multiple chemoresistance. Thus, we used several first-line chemotherapeutic drugs for breast cancer, including 5-FU, doxorubicin (Dox), paclitaxel (Pac), cisplatin (Cis), gemcitabine (Gem), and vincristine (VCR), to examine the multiple chemoresistance of TR cells. Compared to WT cells, TR breast cancer cells
LINC00589 expression is associated with response to trastuzumab in HER2-positive breast cancer. A mRNA expression of LINC00589 in trastuzumab-responding (N = 38) and non-responding (N = 33) breast cancer patients was detected by qRT-PCR. Data were analyzed by a two-tailed t test. B ROC curve for LINC00589 expression to differentiate responding patients from non-responding patients. C The rate of trastuzumab-response patients was significantly higher in the high LINC00589 expression groups than in the low expression group. D In situ hybridization (ISH) staining of high and low expression of LINC00589 in formalin-fixed and paraffin-embedded tissues from HER2-positive breast cancer patients with trastuzumab treatment. Scale bar 50 μm. E Kaplan–Meier’s correlation analyses between LINC00589 expression and overall survival of patients (low, N = 43; high, N = 49). F The expression of LINC00589 in TR clone cells and WT cells was evaluated by qRT-PCR. Assays were conducted in triplicate. Data are shown as the means ± SD; data were analyzed by ANOVA and two-tailed t test. **P < 0.01, ***P < 0.001.

displayed less sensitivity to each of these drugs (Fig. 3F). However, overexpression of LINC00589 remarkably re-sensitized the TR cells to all of these drugs (Fig. 3G). Consistently, we determined HER2 expression by qRT-PCR and western blot assays (Supplementary Fig. 7a–c), and observed decreased HER2 expression in TR cell lines. Meanwhile, LINC00589 overexpression could not change HER2 expression at both mRNA and protein levels in trastuzumab-resistant breast cancer cells (Supplementary Fig. 7d–f). This suggested that LINC00589 might exerted multiple functions through a HER2-independent mechanism in HER2-positive breast cancer. Consistently with the in vitro experiment, we also observed no correlation between LINC00589 and HER2 expression...
in patients’ tissues in clinical samples (Supplementary Fig. 7g). Taken together, the above findings suggest that LINC00589 decreases CSC-like properties and reverses the resistance of TR cells to multiple chemotherapeutic agents.

**LINC00589 functions as a ceRNA and sponges miR-100 and miR-452 in breast cancer cells**

Functional roles of IncRNAs are associated with their cellular localization. To distinguish potential molecular mechanisms whereby LINC00589 exerts its multiple functions in HER2-positive breast cancer, we determined its cellular location. The online IncLocator software predicted that LINC00589 is mainly enriched in the cytoplasm (Fig. 4a). Consistently, subcellular fractionation assays revealed that LINC00589 is mostly distributed in the cytoplasm in both BT474 and SKBR3 cells (Fig. 4b, c). These results raise the possibility that LINC00589 might regulate target protein expression at the post-transcriptional level. As ceRNA mechanism is an important mode for cytoplasmic IncRNA-mediated post-transcriptional regulation\(^\text{22}\), we hypothesized that LINC00589 may competitively sponged miRNAs. To test this hypothesis, we performed an immunoprecipitation assay for Ago2, an important protein component of the RNA-induced silencing complex. The results demonstrate that LINC00589 bind to with Ago2 and was involved in the miRNA-mediated repression of mRNA (Fig. 4d). To further investigate the miRNAs that may be sponged by LINC00589, we used IncBase and obtained 1597 potential binding miRNAs for LINC00589. As we have previously performed a microarray between WT and TR SKBR3 cells (GSE47011)\(^\text{1}\), we selected the most highly upregulated miRNAs (fold change >4.0) and evaluated overlap with IncBase-predicted miRNAs, which yielded 9 candidate miRNAs, including miR-100 (miR-100-5p), miR-7 (miR-7-5p), miR-452 (miR-452-5p), miR-224 (miR-224-5p), miR-4288, miR-3926, miR-151a-5p, miR-17-5p, and miR-125b (miR-125b-5p) (Fig. 4e). Among these 9 candidate miRNAs, only miR-100 and miR-452 mimics were found to suppress LINC00589-driven luciferase activity (Fig. 4f). Therefore, we pursued these two miRNAs as candidates for further investigation.

To verify that miR-100 and miR-452 can interact with LINC00589, we designed reporter constructs in which the putative miR-100 and miR-452-binding sites in LINC00589 were mutated by site-directed mutagenesis (Fig. 4g). As expected, miR-100 and miR-452 decreased the luciferase activity encoded by the WT LINC00589 vector, whereas, mutations of the binding sites abolished their suppressive effect (Fig. 4h, i). To verify that these miRNAs directly bind LINC00589, we performed MS2 pull-down assays using lysates from WT cells and qRT-PCR confirmation (Supplementary Fig. 8a, b). MS2-LINC00589 precipitated miR-100 and miR-452, but MS2-LINC00589-Mut (with mutation of miR-100 and miR-452 binding sequences) failed to enrich these miRNAs, thus suggesting that LINC00589 directly binds to miR-100 and miR-452 through complementary sequences (Fig. 4j). In addition, LINC00589 overexpression decreased levels of miR-100 and miR-452 in TR cells but LINC00589 knockdown increased levels of miR-100 and miR-452 in WT cells (Fig. 4k, l). Altogether, these data indicate that LINC00589 serves as a platform to sponge miR-100 and miR-452 in breast cancer cells.

**miR-100 and miR-452 concurrently regulate trastuzumab resistance, cancer stem cell-like properties, and multidrug resistance**

Next, we explored the functional roles of miR-100 and miR-452, which are directly sponged by LINC00589 in HER2-positive breast cancer cells. qRT-PCR data suggest that expression of both miR-100 and miR-452 is much higher in TR cells than in WT cells (Fig. 5A). To determine whether miR-100 and miR-452 may also modulate trastuzumab resistance, we constructed lentiviruses expressing shRNAs for miR-100 and miR-452 (Supplementary Fig. 9a, b). After trastuzumab treatment, miR-100 or miR-452 knockdown suppressed the viability of TR cells (Fig. 5B, C) and increased

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**Table 1.** Association of LINC00589 expression with clinicopathological features in trastuzumab-treated HER2-positive breast cancer patients.

| Characteristics | Patients with FFPE tissue (n = 92) | LINC00589 \(\chi^2\) | \(P\) |
|-----------------|-----------------------------------|----------------|-----|
| Age             |                                    |                |     |
| <50             | 29                                 | 0.076          | 0.783|
| ≥50             | 63                                 | 0.050          | 0.824|
| Menopausal status|                                  |                |     |
| Pre             | 22                                 | 0.086          | 0.769|
| Post            | 30                                 | 0.086          | 0.769|
| Histological grade|                                |                |     |
| Grade I–II      | 38                                 | 0.887          | 0.356|
| Grade III       | 14                                 | 0.887          | 0.356|
| TNM stage       |                                    |                |     |
| Tu I–II         | 36                                 | 0.887          | 0.356|
| Tu III          | 16                                 | 0.887          | 0.356|
| Lymph node status|                                |                |     |
| Negative        | 25                                 | 0.887          | 0.356|
| Positive        | 27                                 | 0.887          | 0.356|
| ER status       |                                    |                |     |
| Negative        | 10                                 | 0.887          | 0.356|
| Positive        | 42                                 | 0.887          | 0.356|
| PR status       |                                    |                |     |
| Negative        | 23                                 | 0.887          | 0.356|
| Positive        | 29                                 | 0.887          | 0.356|

FFPE formalin-fixed and paraffin-embedded, lymph node status: negative, number of nodal metastases ≤3; positive, number of nodal metastases >3, ER estrogen receptor, PR progesterone receptor. The \(\chi^2\) test was used to compare percentages or the association between LINC00589 and clinicopathological parameters. *\(P < 0.05\)

**Table 2.** Multivariate analysis for breast cancer patients with trastuzumab treatment.

| Characteristics | Overall survival | \(P\) | HR (95% CI) |
|-----------------|-----------------|------|-------------|
| Age             | 0.840           | 1.071| (0.550–2.084)|
| Menopausal status| 0.135          | 0.609| (0.318–1.166)|
| Histologic grade| 0.440           | 0.760| (0.379–1.523)|
| TNM stage       | 0.607           | 1.218| (0.574–2.585)|
| Lymph node status| <0.001         | 3.885| (1.880–8.026)|
| PR status       | 0.925           | 1.045| (0.420–2.601)|
| LINC00589 expression| 0.531        | 0.812| (0.424–1.558)|

HR hazard ratio, lymph node status: negative, number of nodal metastases ≤3; positive, number of nodal metastases >3, ER estrogen receptor, PR progesterone receptor, CI confidence interval. Data were analyzed by cox regression.
the apoptosis rate of TR cells (Fig. 5D, E). In addition, soft agar colony formation assays revealed that the anchorage-independent growth of TR cells with miR-100 or miR-452 knockdown were dramatically lower than those control groups (Fig. 5F, G). These results suggest that miR-100 and miR-452 are involved in trastuzumab resistance in breast cancer.

Further, we investigated the functional roles of miR-100 and miR-452 in CSC-like properties and multiple chemoresistance of HER2-positive breast cancer. The mammosphere-formation data showed that the numbers and volumes of spheres were decreased in TR cells infected with sh-miR-100 and sh-miR-452 lentiviruses as compared to sh-NC lentivirus (Fig. 5H–J). Consistently, sh-miR-100 and sh-miR-452 upregulated the negative stemness marker CD24, and downregulated the positive stemness markers CD44, CD133, Nanog, OCT4, and SOX2, at both the mRNA and protein expression levels (Fig. 5K, L). Finally, the sensitivities of TR cells to 5-Fu, Dox, Paclitaxel, Cisplatin, Gemcitabine and VCR were each increased by miR-100 and miR-452 knockdown (Fig. 5M). Taken together, the above results suggest that miR-100 and miR-452 concurrently regulate trastuzumab resistance, CSC-like properties and multiple chemoresistance in HER2-positive breast cancer cells.

Fig. 2 LINC00589 promotes the sensitivity of breast cancer cells to trastuzumab and inhibits anchorage-independent growth. A–C TR SKBR3 cells were infected with NC or LINC00589-overexpression lentivirus and treated with 25 μg/ml trastuzumab. WT SKBR3 and BT474 cells were infected with sh-NC or sh-LINC00589 lentivirus and treated with 5 μg/ml trastuzumab treatment. The relative cell viabilities were determined by CCK-8 assays at the indicated times. D, E TR and WT SKBR3 cells were infected with overexpression or shRNA lentiviruses and then were treated with 25 μg/ml trastuzumab or 5 μg/ml trastuzumab for 48 h, followed by FITC-conjugated annexin V and PE-labeled PI staining. The apoptosis rate was analyzed by flow cytometry. F, G TR or WT SKBR3 cells were cultured in soft agar for 21 days after trastuzumab treatment and then subjected to crystal violet staining. The clones were observed, and the number of clones for each sample was calculated. Scale bar 100 μm. Data are represented as the mean ± SD of three replicates or are representative of three independent experiments. Two-way ANOVA were used to analyze the data in (A–C), and two-tailed t test was used to analyze the data in (E, G). *P < 0.05, **P < 0.01, and ***P < 0.001 versus negative control (NC).
DLG5 and PRDM16 are direct targets of miR-100 and miR-452 and are indirectly regulated by LINC00589

The biological functions of miRNAs rely on their targets that could be bound with and silenced via the RNA-induced silencing complex. Haven demonstrated the ceRNA interactions of LINC00589-miR-100 and LINC00589-miR-452, We sought to identify downstream targets of miR-100 and miR-452 to elucidate the mechanism through which LINC00589 exerted its functions in TR cells. First, we identified potential targets from the human genome using the bioinformatic miRNA target prediction tools, including miRwalk, Starbase, RNA22, PITA, miRDB, and TargetScan. Among the predicted targets, Discs large homolog 5 (DLG5) for miR-100 and PR/SET domain 16 (PRDM16) for miR-452 drew our attention for the following reasons. DLG5 has been reported to be a suppressor of CSC-like properties in breast cancer cells\(^ {22} \); and PRDM16 functions as a tumor suppressor by inhibiting the transcription of mucin4 (MUC4), which is associated with trastuzumab resistance and CSC-like properties of cancer cells\(^ {23,24} \). To validate these predicted targets for miR-100 and miR-452, we constructed wild-type (WT) and mutant (Mut) dual-luciferase reporter vectors for DLG5 and PRDM16. qRT-PCR results confirmed that miR-100 and miR-452 significantly inhibited WT but not Mut DLG5 and PRDM16 luciferase activity, respectively (Fig. 6b, c). To further investigate the regulatory effects of miR-100 and miR-452 on DLG5 and PRDM16 expression, we performed qRT-PCR and
western blotting analyses, which revealed that miR-100 mimic decreases DLG5 mRNA and protein expression (Fig. 6d, e), and miR-452 mimic decreases PRDM16 mRNA and protein expression (Fig. 6f, g). These results suggest that DLG5 and PRDM16 are direct targets for miR-100 and miR-452.

Next, we investigated whether LINC00589 regulates DLG5 and PRDM16 via miR-100 and miR-452. Knockdown of LINC00589 decreased DLG5 expression, whereas miR-100-suppression abolished the inhibitory effect of LINC00589 silencing on DLG5 expression in WT cells, as confirmed at both the mRNA (Fig. 6h) and protein levels (Fig. 6i). Furthermore, knockdown of LINC00589 suppressed PRDM16 expression, which was rescued by miR-452 inhibition (Fig. 6j, k). These results suggested that LINC00589 regulated DLG5 and PRDM16 via miR-100 and miR-452, respectively. Further, we constructed PRDM16 overexpression vector to confirm the regulatory effect of PRDM16 on MUC4 levels in breast cancer cells, and the efficiency was tested by qRT-PCR assay (Supplementary Fig. 10a). MiR-452 mimic...
enhanced MUC4 expression, whereas PRDM16 overexpression abolished the miR-452-induced upregulation of MUC4 in WT cells (Fig. 6f, m). These results provide further evidence for MUC4 as a downstream target in the LINC00589-miR-452-PRDM16 axis. Collectively, our results identified the LINC00589-miR-452-DLG5 and the LINC00589-miR-452-PRDM16-MUC4 axes in breast cancer.

LINC00589 regulates trastuzumab resistance, cancer stem cell-like properties, and multiple chemoresistance of breast cancer via DLG5 and PRDM16

We next investigated whether LINC00589 exerts its biological functions through DLG5 and PRDM16. Overexpression efficacy of pCDNA-DLG5 (Supplementary Fig. 10b), and knockdown efficacy of sh-PRDM16 and sh-DLG5 were determined by qRT-PCR (Supplementary Fig. 10c, d). The CCK-8 assays demonstrated that knockdown of LINC00589 increased the viability of WT SKBR3 and BT474 cells under trastuzumab treatment, but that overexpression of either DLG5 or PRDM16 abolished the increased cell viability induced by sh-LINC00589 (Fig. 7A, B). In contrast, the viability of TR cells was suppressed by LINC00589, while sh-DLG5 or sh-PRDM16 co-transfection abolished the decreased cell viability mediated by LINC00589 (Fig. 7C). Consistently, knockdown of DLG5 and PRDM16 also partially abolished LINC00589-enhanced cell apoptosis after trastuzumab treatment (Fig. 7D, E). Moreover, soft agar colony formation assays revealed that silencing of DLG5 or PRDM16 abrogated LINC00589-induced inhibition of anchorage-independent growth in TR cells (Fig. 7F, G). These data indicate that LINC00589 reverses trastuzumab resistance by regulating DLG5 and PRDM16.

To confirm the roles of DLG5 and PRDM16 in LINC00589-regulated CSC-like properties and multiple chemoresistance of breast cancer, we performed mammosphere formation and cell viability assays. The number and volume of spheres were downregulated by LINC00589 in TR cells, while knockdown of either DLG5 or PRDM16 partially abated the suppressive effect of LINC00589 (Fig. 7H–j). Furthermore, qRT-PCR and western blot analyses revealed that knockdown of DLG5 or PRDM16 partially relieved the stimulatory effect of LINC00589 on the expression of the negative stemness marker, CD24, and antagonized the repression of LINC00589 on the expression of the positive stemness markers, CD44, CD133, Nanog, OCT4, and SOX2 (Fig. 7K, L). Finally, cell viability assays demonstrated that silencing of DLG5 or PRDM16 partially abrogated LINC00589-induced sensitization of TR cells to 5-FU, Dox, Pac, Cis, Gem, and VCR (Fig. 7M). Collectively, these results indicate that LINC00589 regulates trastuzumab resistance, cancer stem cell-like properties and multiple chemoresistance, at least in part, by modulating DLG5 and PRDM16 expression.

Fig. 4 LINC00589 sponges miR-100 and miR-452. a LINC00589 localization was predicted using the lncRNA subcellular localization predictor IncLocator. b, c LINC00589 localization was confirmed by subcellular fractionation assays in BT474 and SKBR3 cells. Nuclear control: U6; cytosolic control: GAPDH. d RNA experiments were performed in BT474 and SKBR3 cells, and the coprecipitated RNA was used to quantify LINC00589 expression by qRT-PCR. e Potential miRNAs binding to LINC00589 via miRNA-LINC00589 interaction were predicted by lncBase. Then the predicted miRNAs and the upregulated miRNAs in TR cells from GEO dataset (GSE47011) were overlapped. Nine candidate miRNAs were obtained for further investigation. f Luc-LINC00589 vector was co-transfected with the NC mimic (MIM) or the nine miRNAs MIM into TR cells for 48 h, followed by luciferase reporter assay. g Predicted miR-100 and miR-452 binding sites on LINC00589 and the mutations for luciferase reporter assay are shown. h, i Luciferase reporter plasmids containing wild type (WT) or mutant (Mut) LINC00589 were co-transfected with NC MIM, miR-100 MIM, or miR-452 MIM into TR cells for 48 h. Subsequently, the luciferase activity was evaluated. j MS2, MS2-LINC00589 or MS2-LINC00589 harboring mutations (Mut) in the miR-100 or miR-452 binding sites were transfected into WT cells for 48 h. Then, total RNA was incubated with MBP-MCP-conjugated amylose resin, and the immunoprecipitated RNA was subjected to qRT-PCR. k Expression of miR-100 and miR-452 in TR cells infected with Lv-NC or Lv-LINC00589 in WT cells infected with sh-NC or sh-LINC00589 was measured by qRT-PCR assay. U6 was used as an internal control. Data are shown as mean ± SD; two-tailed t test was used to analyze the data in (d, f, h, i, j, k, and l). *P < 0.05, **P < 0.01 and ***P < 0.001 versus negative control (NC).

LINC00589-initiated ceRNA networks serve as key regulators of trastuzumab resistance in vivo

To investigate the functional role of the LINC00589-initiated ceRNA networks in trastuzumab resistance in vivo, we established an animal model of nude mice bearing TR breast cancer cell xenografts. TR cells infected with Lv-LINC00589 or Lv-NC were implanted into mammary fat pads of nude mice, which were divided into four groups: group A (Lv-NC + miR-NC), group B (Lv-LINC00589 + miR-NC), group C (Lv-LINC00589 + miR-100) and group D (Lv-LINC00589 + miR-452). When the xenograft volumes reached 50 mm³, miR-100 mimic, miR-452 mimic or control miRNA were injected into the tumors (15 μg /injection, twice a week) before injection of trastuzumab (10 mg/kg). Consistent with our in vitro results, LINC00589 overexpression significantly decreased the tumor volume and weight; however, either miR-100 or miR-452 reversed the repressive effect of LINC00589 on tumor volume and weight in nude mice (Fig. 8A–C). To further validate the effect of LINC00589 on trastuzumab resistance in vivo, we injected TR cells after stable transfection of lentivirus-NC-Luc or lentivirus-LINC00589-Luc. The results demonstrate that LINC00589 inhibited the luciferase activity of tumors, but both miR-100 and miR-452 abated LINC00589-induced suppression of tumor growth (Fig. 8D–F). These in vivo data suggested that LINC00589 reversed trastuzumab resistance via miR-100 and miR-452 in breast cancer in vivo.

Next, we explored the downstream activation of LINC00589-initiated ceRNA networks in vivo. Cancer tissues from xenografts were dissected and subjected to RNA isolation and qRT-PCR. Compared to the control vector, LINC00589 decreased the mRNA expression of miR-100, miR-452, and increased the mRNA expression of miR-100, DLG5 and PRDM16. However, miR-100 abolished the effect of LINC00589 in promoting mRNA expression of DLG5, and miR-452 abated the enhancement of LINC00589 on mRNA expression of PRDM16 (Fig. 8J, K). Moreover, IHC assay data in xenograft tumor tissues revealed that LINC00589 overexpression could upregulate the protein expressions of DLG5 and PRDM16, while miR-100 and miR-452 abrogated the promotion of LINC00589 on the DLG5 and PRDM16, respectively (Fig. 8L). Collectively, these results confirm the LINC00589-initiated ceRNA networks and their downstream targets in vivo.

LINC00589-initiated ceRNA networks are clinically relevant in HER2-positive breast cancer in the clinic

To further evaluate the relevance of the LINC00589-miR-100-DLG5 and LINC00589-miR-452-PRDM16-MUC4 networks in clinical samples, we evaluated expression levels in HER2-positive breast cancer patients. The mRNA levels of LINC00589 were negatively correlated with mRNA levels of both miR-100 and miR-452, and mRNA levels of miR-100 and miR-452 were conversely correlated with DLG5 and PRDM16, respectively (Fig. 9A–D). Moreover, IHC assay demonstrated low expression
of DLG5 and PRDM16 in trastuzumab non-response HER2-positive breast cancer patients but high expression of DLG5 and PRDM16 in trastuzumab-response HER2-positive breast cancer patients (Fig. 9E). These results confirmed the clinical relevance of the LINC00589-initiated ceRNA networks in breast cancer patients in clinic.
Increasing evidence has suggested that drug resistance, EMT and CSC-like properties, which are important causes of cancer progression, may function concordantly. Therefore, the identification of molecular signatures that concurrently regulate these processes holds great significance for cancer characterization, therapy and prognosis evaluation. Here, we provided the evidence that LINCO0589 concurrently reverses trastuzumab resistance, MDR and CSC-like properties and serves as an independent prognostic factor in HER2-positive breast cancer. Further mechanistic investigation revealed that LINCO0589 exerts its functions via two axes, the mir-100/DLG5 and mir-452/PRDM16 axis, as ceRNA.
Fig. 7 LINC00589 represses trastuzumab resistance, cancer stem cell-like properties, and multiple chemoresistance via DLG5 and PRDM16. A–E WT SKBR3 and BT474 cells infected with sh-LINC00589 or sh-NC lentivirus were transfected with pCDNA (pC) -DLG5 or pC-PRDM16 for 48 h; or TR SKBR3 cells infected with Lv-LINC00589 or the Lv-NC were transfected with sh-DLG5 or sh-PRDM16 for 48 h. The relative cell viabilities of WT SKBR3 cells (A), BT474 cells (B) and TR SKBR3 cells (C) were quantified by CCK-8 assay. Apoptosis of TR cells were examined by flow cytometry assay (D) and the apoptosis rate was calculated (E).

F–M LINC00589- or NC- overexpressing TR cells were infected with sh-NC, sh-DLG5 or sh-PRDM16 lentivirus. Cells were cultured in soft agar for 21 days, representative images of colony formation were observed (F), and the number of colonies were calculated (G). Scale bar 100 μm. Cells were seeded in an ultra-low-attachment culture system. Representative images of mammosphere formation were observed (H), and numbers (I) and volumes (J) of mammospheres were calculated. Scale bar 100 μm.

Data are shown as the mean ± SD of five random high-power fields (HPF) and were analyzed by two-tailed t test. *P < 0.05, **P < 0.01, and ***P < 0.001 versus negative control (NC).
Fig. 8  Function of LINCO0589 initiated ceRNA networks in vivo. A Nude mice were subcutaneously injected with different TR breast cancer cells with different treatments, including Lv-NC, Lv-LINC00589, miR-NC, miR-100 mimic, or miR-452 mimic, (n = 5). Images of tumors dissected from four groups of nude mice at the end of the experiment are shown. B The tumor volume was recorded at the indicated days. C Final weights of tumors resected from all groups of sacrificed mice. D–F Nude mice were implanted with the indicated TR cells (n = 5). Whole-body fluorescent images were obtained at the indicated time intervals after injection (D), the luciferase activity at different times (E) and at the end (F) was calculated. Data are represented as mean ± SD and were analyzed by two-way ANOVA. G–K Expression of LINC00589 (G), miR-100 (H), miR-452 (I), DLG5 (J), and PRDM16 (K) in tumor tissues from the xenografts were determined by qRT-PCR assay. L Protein expression of DLG5 and PRDM16 in tumor tissues from the xenografts were analyzed by immunohistochemistry (IHC). Represent images are shown. Scale bar 50 μm. Data are shown as mean ± SD; two-tailed t test was used to analyze the data in (J, K). *P < 0.05, **P < 0.01, and ***P < 0.001 versus negative control (NC).
platforms (Fig. 10). These data uncover new signaling networks that underlie the crosstalk between trastuzumab resistance, MDR and CSC-like properties in breast cancer.

HER2-targeted therapy is a standard treatment for early or metastatic HER2-positive breast cancer and often improves clinical outcomes; however, primary and acquired resistance occurs in a substantial subset of patients. Many efforts have been made to elucidate the mechanisms of trastuzumab resistance, mainly including but not limited to: (a) HER2 loss, extracellular domain-deficient P95 HER2 expression and MUC4 masking have been demonstrated to block the access of trastuzumab to HER225. (b) High expression of the Delta16 HER2 isoform was shown to mediate optimal efficacy for trastuzumab 26. (c) Activation of downstream effectors of HER2 signaling (e.g., PTEN) and alternative signaling pathways (e.g., IGF1R signaling) lead to trastuzumab resistance in breast cancer27. (d) Breast cancer cells have been demonstrated to escape from antibody-dependent cell-mediated cytotoxicity (ADCC) caused by trastuzumab28. These findings imply that trastuzumab resistance may arise from a combination of largely unknown mechanisms. In addition, emerging evidence shows that trastuzumab resistance is not an isolated phenomenon but is often accompanied by MDR and CSC-like properties18. However, other potential key regulators that link these processes and their relationships have not been well explored.

Noncoding RNAs, most of which have not been functionally annotated, play central roles in various physiological and pathological processes, especially in complicated signaling networks of cancers29,30. Our previous studies and other reports demonstrate that noncoding RNAs also regulate trastuzumab resistance to breast cancer, e.g., miR-200c, miR-221, miR-375, lncRNA TINCR, AGAP2-AS1, and UCA11,3,10,11,29,30. LINC00589, also named as TSLNC8, was identified on chromosome 8p12 by Strausberg et al.31. Until recently, functional roles of LINC00589 began to get much concern. For example, LINC00589 serves as a tumor suppressor in human glioma32 and non-small cell lung cancer15, and inhibits melanoma resistance to BRAF inhibitor33. However, LINC00589 also interacts with HUR and stabilizes CTNNB1 mRNA, thereby promoting cancer progression in pancreatic cancer13. Thus, LINC00589 may serve as both a tumor suppressor and a tumor promoter according to different cancer pathological settings. In this study, we determined that LINC00589 is downregulated in trastuzumab-resistant breast cancer and serves as an independent prognostic factor for HER2-positive patients. Furthermore, LINC00589 concurrently reversed trastuzumab resistance, multiple chemoresistance, and CSC-like properties.
LINC00589-dominated two novel ceRNA networks in breast cancer

Giusti et al. disclosed that HER2 loss also results in both trastuzumab resistance and enhanced stemness of breast cancer, which is consistent with our findings and indicates HER2 loss probably is likely to correlate with LINC00589. However, LINC00589 is unlikely to regulate HER2 expression in breast cancer and instead exerts multiple functions in trastuzumab resistance, MDR and CSC-like properties through a HER2-independent mechanism.

LncRNAs, miRNAs, and mRNAs have been shown to crosstalk with each other through shared binding sequences within complex signaling networks via ceRNA mechanisms. Emerging evidence indicates that LncRNAs serve as sponges for miRNAs and thereby keep them away from binding sites on target genes in various cancer types.

For example, LncRNA DNACR enhances ROCK1-mediated proliferation and metastasis by sponging miR-335-5p and miR-197 in osteosarcoma. LncRNA LINC01123 sponges miR-199a-5p and triggers proliferation and aerobic glycolysis by regulating c-myc expression in non-small cell lung cancer. LINC00673, which is upregulated by YY1, exerts oncogenic functions in breast cancer by sponging miR-515-5p and subsequently upregulates MARK4 expression, and inhibits the Hippo signaling pathway. In this study, we demonstrate that LINC00589 is mainly localized to the cytoplasm, which is consistent with the possibility that it may function as an endogenous miRNA sponge. Bioinformatics analysis and experimental assays further revealed that miR-100 and miR-452 are direct targets of LINC00589. MiR-100 and miR-452 have been reported as both oncogenes and tumor suppressor genes. For example, miR-100 promotes cetuximab resistance in colorectal cancer but inhibits bladder urothelial carcinogenesis, and miR-452 promotes renal cancer cell invasion and metastasis and colorectal cancer progression, but inhibits metastasis of non-small cell lung cancer. In our study, downregulation of both miR-100 and miR-452 suppressed trastuzumab resistance, multiple chemoresistance, and CSC-like properties, thus supporting their oncogenic roles in HER2-positive breast cancer as targets of LINC00589.

According to the ceRNA network theory, roles for LncRNAs are dependent on their abilities to regulate miRNA targets that mediate signaling pathways. Therefore, investigating potential targets of miRNAs is important for elucidating the roles and mechanisms of ceRNA networks. Consistently, in this study we identified DLG5 as a target of miR-100 and PRDM16 as a target of miR-452. DLG5 belongs to the membrane-associated guanylate kinase (MAGUK) superfamily and is considered to play multiple roles in various cancers, including an ability to suppress breast cancer stem cell-like characteristics and restore tamoxifen sensitivity by inhibiting TAZ expression and to decrease the formation and function of invadopodia in human hepatocellular carcinoma via Girdin and Tks5. On the other hand, PRDM16, a zinc finger transcription factor hammering the epithelial-to-mesenchymal transition, functions as a suppressor of lung adenocarcinoma metastasis and is associated with patient survival. In kidney cancer, PRDM16 suppresses HIF-targeted gene expression and inhibits tumor growth in vitro and in vivo.
In this study, we identified DLG5 and PRDM16 as target genes for miR-100 and miR-452. Thus, our results support the ability of LINCO0589 to regulate both miR-100/DLG5 and miR-452/PRDM16 axes, thereby suggesting two crosslinked ceRNA pathways. In support of this possibility, we demonstrated that silencing of either DLG5 or PRDM16 abolished multiple LINCO0589-induced effects in HER2-positive breast cancer.

In conclusion, we demonstrated that LINCO0589 concurrently reverses trastuzumab resistance, multiple chemoresistance and CSC-like properties and acts as an independent prognosis factor for HER2-positive breast cancer. Further, we identified that two ceRNAs networks, LINCO0589-miR-100-DLG5 axis and LINCO0589-miR-452-PRDM16 axis, that mediate multiple suppressor roles of LINCO0589. Our findings suggest that these LINCO0589 ceRNA networks could be valuable for predicting trastuzumab efficacy and prognosis, as well as providing promising therapeutic targets for HER2-positive breast cancer in future translational applications.

METHODS

Patient samples

A total of 71 cases of trastuzumab-treated HER2-positive breast cancer patients were enrolled from General Hospital of Xinjiang Command and Xijing Hospital before chemotherapy was initiated. Cases with complete response (CR) or partial response (PR) were considered as trastuzumab responders, and cases with stable disease (SD) or progressive disease (PD) were defined as trastuzumab non-responders. Clinical tissue samples were obtained during the operation and were immediately frozen at −80 °C until RNA extraction. Another independent cohort of 92 cases of paraffin-embedded samples from HER2-positive breast cancer patients who received trastuzumab were obtained from the General Hospital of Xinjiang Command and Xijing Hospital. Ethical approval was obtained from the Ethics Committee of the General Hospital of Xinjiang Command (number: 201803). All participants provided written informed consent. The detailed clinicopathological characteristics of these paraffin-embedded samples are summarized in Table 1. All patients were pathologically confirmed for diagnosis of HER2-positive breast cancer.

Reagents

Trastuzumab (Herceptin) was purchased from Roche (Basel, Switzerland) and dissolved in phosphate-buffered saline (PBS). 5-Fluorouracil (5-FU), doxorubicin (Dox), paclitaxel (Pac), cisplatin (Cis), gemcitabine (Gem), and vincristine (VCR) were obtained from Sigma-Aldrich (St Louis, MO, USA).

Cell lines and cell culture

BT474 human breast cancer cells (HER2-overexpression) were obtained from the American Type Culture Collection (catalog number HTB-20, ATCC) and were cultured in RPMI 1640 supplemented with 10% FBS. Wild-type (WT) SKBR3 human breast cancer cells (catalog number HTB-30, ATCC) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Trastuzumab-resistant (TR) SKBR3 cells were established by continuous culture of WT SKBR3 cells in the presence of 5 μg/ml trastuzumab for 6 months in a humidified atmosphere of 5% CO2 and 95% air at 37 °C according to our previous reports1,3.

Cell transfection

The full-length coding sequences of DLGS and PRDM16 were amplified and cloned into the pCDNA3.1 overexpression vector (catalog number V79520, Invitrogen, Carlsbad, CA, USA). The control and overexpression vectors were transfected using Lipofectamine 2000 (catalog number 11668019, Invitrogen, Carlsbad, CA, USA) at a 1 μg DNA: 2.5 μl lipofectamine ratio according to the manufacturer’s instructions. miRNA mimics were synthesized by Shanghai Gene Pharma Co, Ltd. The target sequences were as follows: miR-100: 5′-AACCCGUAUGCAGCACCAUGUG-3′; miR-452: 5′-AACUGUUUGACAGGAAACUGA-3′; miR-7: 5′-UGGAGACGACUGAUAUUGUUUGU-3′; miR-224: 5′-UCAAGACAGCCGUGUCCGUGU-3′; miR-4288: 5′-UUGUGUCUGAGGUUCC-3′; miR-3926: 5′-UGGGCAAAACGAGCAGGAA-3′; miR-151a-5p: 5′-UCGAGGCGCUACAGCUAGU-3′; miR-17-3p: 5′-ACUGCAAGUGAACCUUGAGU-3′; miR-125b: 5′-UCCCUGA GACCCUACUUUGUGA-3′. The working concentrations for miRNA mimics were 30 nM for cell transfection. RNA was transfected into cells using Lipofectamine 2000 according to the manufacturer’s instructions.

Expression vector construction, lentiviral package, and transduction

Full-length coding sequences of DLGS and PRDM16 were amplified and cloned into pcDNA3.1 vector. Full-length coding sequences of LINCO0589 were amplified and cloned into pLVX-Puro vector (catalog number PT4002-5, Clontech, CA, USA) with or without the luciferase gene. RNA oligos containing siRNA sequences of LINCO0589, DLGS, and PRDM16, or containing inhibitor sequences of miR-100 or miR-452 were synthesized and cloned into the shRNA lentivector pLVX-shRNA2 (catalog number PT4052-5, Clontech, CA, USA) with either the puromycin or bleomycin resistance marker gene. The sequences were as follows: siRNA-LINCO0589: 5′-GGATGACACCTCATTCA-3′; siRNA-DLGS: 5′-GCTCAAGGACGACACTC-3′; siRNA-PRDM16: 5′-CCCAACACTTGGTCTCA-3′; miR-100 inhibitor: 5′-CACAGAAGCGAUCGCGGGA-3′; miR-452 inhibitor: 5′-UCAAGG-GUUUCUCUGGACAACTTT-3′. The retrovirus constructs or the empty vector (control vector) were transiently co-transfected with package vectors into 293T cells to produce lentiviruses, which were collected in the viral supernatant 72 h after transfection. Cells infected with the packaged viruses were pre-treated with DEAE dextran (25 μg/ml) for 45 min. 48 h after the infection, cells were screened with puromycin (2 μg/ml) or bleomycin (100 μg/ml), depending on the selection marker of the vector. Cells infected with multiple constructs were selected for infection with each construct.

Bioinformatic analysis

The sequence of LINCO0589 was downloaded from NCBI (gene ID: 619351), from which a 1413 base pair (bp) sequence was predicted. The secondary structure of LINCO0589 was predicted by AnnoLnc (http://annolnc.cbi.pku.edu.cn/)58. The IncRNA subcellular localization was predicted using the online website IncLocator (http://www.csbio.sjtu.edu.cn/bioinfo/IncLocator/)56. The potential sponged miRNAs for LINCO0589 and their binding sites were predicted by LincBase tools (http://carolina.imis.athena-innovation.gr/diana_tools). Starbase (http://starbase.sysu.edu.cn/), miRWalk (http://mirwalk.unni-heidelberg.de/), RNA22 (https://cm.jefferson.edu/ma22/), PITA (https://genie.weizmann.ac.il/pubs/mir07/), miRDB (http://mirdb.org/), and TargetScan (http://www.targetscan.org/vert_71/), were used to predict the potential target genes of miR-100 and miR-452.

Real-time RT-PCR analysis

Total cell RNA was extracted using Trizol (Invitrogen). cDNA was synthesized from 1.0 μg of total RNA, using oligo-dT priming and the Retroscript reverse transcription kit (Ambion, cat no. AM1710). Real-time PCR was performed in triplicate using the Real-Time SYBR Green PCR master mix system (SuperArray Bioscience Corporation, cat no. PA-110) on an Opticon DNA Monitor instrument (Biorad). mRNA levels were normalized to GAPDH, which was used as an internal control. The primer sequences were as follows: forward primer 5′-CACCGTTGCCAAAGTCAACG-3′ and reverse primer 5′-ACCGGCTTCCCAGAACCACG-3′ for LINCO0589; forward primer

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5'-GGTCTCCTCCCTTTGCTGATAT-3', reverse primer 5'-GGGACACCATCAACGATCCACGACAG-3'; reverse primer 5'-TGTCAAACCTCAGTGGACACTCTTG-3', forward primer 5'-CAGTGGTGACGGGAGACAG-3' for OCA7; forward primer 5'-AACAGCGGCAGGACAGTA-3'; reverse primer 5'-GACCTTGACACAGGGCACCACAT-3' for SOX2; forward primer 5'-CTGCAGACATCAACGTAGGTTTGGG-3', reverse primer 5'-AGGGAGAGGTGCCACCAAGGAGCA-3'; forward primer 5'-GTGTTCTCACTCTCTTCTGCATCTCT-3' for PRDM16; forward primer 5'-TCTGCTCCTGGCAGAGGTCTTGCCGACAG-3', reverse primer 5'-GAACCAACGACGATTGCCGACAT-3' for HER2; and forward primer 5'-CTCTCCACCTTTGTGACCCTG-3', reverse primer 5'-TCCTTCTGCTTCCTTGCT-3' for GAPDH. For miRNAs, the expression levels were normalized to U6 small nuclear RNA (internal control), and the following universal primers from the QIAGEN kit were used: forward primer 5'-AACCAAGCATCAACCGGAGAAGCA-3'; reverse primer 5'-AACCCGTAGATCCGAACTTGTG-3', reverse primer 5'-AATGTGGTCCGAGTGTGGTTTC-3', forward primer 5'-AGGCAAACAACCCACTTCTG-3' for CD44; forward primer 5'-GGAGAAGCTGCAGACCCCTTCTG-3', reverse primer 5'-CAGGGAGACAGGTCTCTCTTGG-3', reverse primer 5'-CGGCCAGCATACACTCCATT-3', forward primer 5'-TCTGAGTTCTCTGCCGTAGGTGTCC-3', reverse primer 5'-GTTCTCTGCCGTAGGTGTCC-3', reverse primer 5'-GCCAGAGTCGCCAGAGAGGTGCCACCAAGGAGCA-3'; forward primer 5'-GCGAGAACCCCAAAACGTGACGGAGACAG-3', reverse primer 5'-CGAGAAGTCCGGCAACAAGGAGGA-3'; and reverse primer 5'-TTGGGACGATGCTAGCACTGGCATC-3', forward primer 5'-TACACAAGCGCAATGACCCACTTCTG-3', reverse primer 5'-GAGGAGAGGTGCCACCAAGGAGCA-3'; forward primer 5'-GAGGAGAGGTGCCACCAAGGAGCA-3'; reverse primer 5'-TTGAGAGGAGGAGAATAAA-3', forward primer 5'-TTTATATTTTTCCCTTCTTGTA-3', and reverse primer 5'-TCTTCCAAAACATAACAGAAATA-3'.

**Dual-luciferase reporter gene assays**

Breast cancer cells were co-transfected with 150 ng of firefly luciferase plasmid with inserted WT and Mut sequences from LINCO00589, DL5S, or PRDM16, together with pRL-SV40 Renilla luciferase vector (catalog number E2231, Promega) and miRNAs (miR-100 mimic, miR-452 mimic or negative control RNA) using Lipofectamine 2000 (Invitrogen). Three independent transfection experiments were performed, each in triplicate. 48 h after transfection, firefly luciferase activity derived from pGL3 plasmids was evaluated and normalized to Renilla luciferase activity using a luciferase assay system (Promega) as reported previously.

**CCK-8 cytotoxicity assay**

Cytotoxicity was analyzed using the cell counting kit-8 (CCK-8) method (MYBiotech, China). Briefly, breast cancer cells were transfected with plasmids and/or miRNA mimics/shRNAs or were infected with lentiviruses. Subsequently, the cells were plated in 96-well plates and exposed to trastuzumab, 5-FU, doxorubicin, paclitaxel, cisplatin, gemcitabine, or vincristine over a time course. After drug treatment, 10 μl CCK-8 solution was added to each well, and the cells were incubated at 37 °C for 4 h. The optical density (OD) was measured at 450 nm (Thermo Scientific, USA), and the half-maximal inhibitory concentration (IC50) of the drug was calculated based on the OD value. The assay was performed at least three times. Cell cytotoxicity was calculated according to the following formula: inhibition ratio (%) = (OD (drug) – OD (blank))/ (OD (drug control) – OD (blank)) × 100%

**Soft agar colony formation assays**

Low melting temperature agarose was mixed with culture medium to obtain 0.6% and 0.35% gel as the “lower” and the “upper” soft agar, respectively. Plates with 6 wells were coated with 1.0 ml lower soft agar. Then 1.0 × 10⁶ cells were resuspended with 2 ml upper soft agar and immediately plated on the lower soft agar. Cells were incubated for 21 days in a 37 °C incubator and were stained with 0.005% Crystal Violet Staining Solution. Colonies were enumerated by microscopy. Experiments were carried out in triplicate and were repeated a minimum of three times.

**Flow cytometry analysis**

WT SKBR3 (catalog number HTB-30, ATCC) and TR SKBR3 breast cancer cells were seeded in six-well plates (5 × 10⁶ cells/well) and were transfected with miRNA mimics or plasmids, or they were
infected with lentiviruses. The cultures were supplemented with trastuzumab at a final concentration of 5 μg/ml for WT SKBR3 cells (catalog number HTB-30, ATCC) or 25 μg/ml for TR SKBR3 cells. Then, the cells were washed three times with PBS, harvested, stained with annexin V-FITC and propidium iodide (BD Biosciences), and subjected to flow cytometry (BD Biosciences) to detect apoptosis.

Subcellular fractionation assay

Seeded breast cancer cells (2 × 10^5 cells) were washed with ice-cold PBS and resuspended in the ice-cold cytoplasmic lysis buffer (0.15% NP-40, 10 mM Tris pH 7.5, 150 mM NaCl) for 5 min on ice. The lysates were transferred into ice-cold sucrose buffer and centrifuged at 13,000 × g for 10 min at 4 °C. The supernatant (~700 μL) was collected as the cytoplasmic fraction and the precipitate was collected as the nuclear fraction. The expression of LINC00589 in different subcellular fractions was analyzed by qRT-PCR.

Mammosphere-formation assay

Breast cancer cells were seeded onto ultra-low attachment six-well plates (3471; Corning, Corning, NY, USA) at a density of 2000 cells per well. The CSCs were cultured for 14 days using the Mammocult Human Medium Kit (Stemcell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. Mammospheres were digested in trypsin/EDTA and centrifuged at 300 × g for 10 min. Then, the cells were resuspended and cultured for the next round of sphere formation. Cells from the sixth-generation spheres were used to analyze the efficiency of mammosphere formation. Formed spheres were counted manually, and representative images were obtained by microscopy.

MS2 RNA pull-down assay

To explore the interactions between LINC00589 and miRNAs, we performed a MS2 RNA pull-down assay, in which the MBP-MCP fusion protein recognizes MS2 hairpins. Breast cancer cells were transfected with MS2, LINC00589-MS2 or LINC00589-Mut-MS2 plasmids and harvested 48 h post-transfection. Then, breast cancer cell lysates were incubated with MBP-MCP-coated amylase resin (prepared at 4 °C) for 8 h. Bound LINC00589-MS2 complexes were eluted with 100 μl buffer containing 20 mM maltose after incubation and extensive washing. The eluted complexes were used to identify LINC00589-associated miRNAs. qRT-PCR analysis was performed to identify the miRNAs associated with LINC00589.

Tumor xenografts and growth measurement

TR breast cancer cells were infected with LINC00589 overexpression or control lentivirus and cultured for cell expansion. Female athymic BALB/c nude mice (4–6 weeks, 20 g) were purchased from the Experimental Animal Center, Chinese Academy of Science (Shanghai, China). Mice were housed in a pathogen-free animal facility at 22 ± 2 °C under controlled 12-h light/dark cycles. Mice were given regular chow or special custom diets when indicated and had access to autoclaved water ad libitum. Animals were grouped by simple randomization using a random number table. To form orthotopic mammary fat pad tumors, the surgical area was depilated and swabbed with 70% ethanol before making an incision in the skin of the breast. Next, 4 × 10^6 cells were subcutaneously injected into the mammary fat pad area in situ. When the volume of xenograft tumors reached 50 mm^3, mice were randomized into two groups. One group was injected with 50 μg/injection, twice a week. Tumor volumes were monitored every 3 days for 7 weeks according to the formula:

\[ \text{tumor volume (mm}^3\) = length \times width^2/2. \]

To further investigate the function of LINC00589-initiated ceRNA networks in vivo, another group of nude mice were injected with LINC00589-Luc and control Luc lentivirus-infected TR breast cancer cells and were administered the same treatments described above. Five minutes after administration of 1.5 mg luciferin (Gold Biotech, St Louis, MO, USA), the luciferase activity of tumor xenografts was monitored using an IVIS® Lumina II system (Caliper Life Sciences, Hopkinton, MA, USA), which was repeated every 3 days. At the end of the experiments, all mice were euthanized by amobarbital injection of three times standard doses, and the tumor tissues were isolated and snap-frozen for miRNA expression analysis. The investigators had no bias and special tendency in the processing of animal experiments. All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of the General Hospital of Xinjiang Command and were approved by the local animal experiments ethical committee.

In situ hybridization (ISH) staining assay

Expression of LINC00589 in paraffin-embedded breast cancer tissues was determined by in situ hybridization (ISH) experiments. Briefly, after dewaxing and rehydration, the paraffin-embedded breast cancer tissues were digested with 10% trypsin for 40 min at room temperature and then were hybridized with the digoxin-modified LINC00589 probe (5’-TACTGTCTCTCCTCGGAGCAGGATTC CATCTTT-3’, Exiqon, Vedbaek, Denmark) at 55 °C overnight, followed by incubation with antibody against digoxin (Roche) and staining. ISH signals for LINC00589 expression were determined in the form of the mean optical density using the AxioVision Rel.4.6 computerized image analysis system. The staining index (SI) was determined based on both the intensity and proportion of LINC00589. The expression of LINC00589 was evaluated using the SI and scored as 0, 1, 2, 3, 4, 5, or 6. LINC00589 expression was defined as high (SI ≥ 4) or low (SI < 4) based on the distribution of the frequency of SI scores.

Statistics analysis

Data were analyzed using SPSS 19.0 software for windows. The results are presented as the mean ± SD. Statistical analysis was performed using Student’s t test or analysis of variance (ANOVA) to compare means of the two groups or multiple groups of in vitro and in vivo data. The χ2 test was used to compare percentages or the association between LINC00589 and clinicopathological parameters. Multivariate Cox regression was used to analyze independent prognostic factors for overall survival in HER2-positive breast cancer patients. The Spearman correlation test was performed to identify the correlation between the mRNA expression of target genes. Receiver Operating Characteristic (ROC) curve analysis was conducted, and the cutoff value was used to discriminate trastuzumab-responding or non-responding HER2-positive breast cancer patients. All data graphs were drawn using the PRISM Software, Version 9 (GraphPad Software, CA, USA). A value of P < 0.05 was considered statistically significant.

DATA AVAILABILITY

The microRNA microarray data of breast cancer tissues have been deposited in NCBI Gene Expression Omnibus database (GEO) and are openly available via accession https://identifiers.org/geo: GSE47011. The data generated and analyzed during this study are described in the following data record: https://datadryad.org/stash/share/V-yCLVwMfsd1VQkCILYaSC51DxshalCmD71UeYkVc, or contacted to QH Ji to access this data. For clinical research purposes, this data is anonymized. Files underlying the clinical data are openly available in Excel format. All other data supporting the study can be found in the supplementary information file, and the corresponding author can make any materials available upon request. Un-cropped gels and western blots for Figs. 1–7 are included in supplementary materials (Supplementary Fig. 11). Western blot quantifications are included in supplementary materials.
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AUTHOR CONTRIBUTIONS
Q.H.J., W.D.B., H.Y.Z., and Y.Z.L. developed the original hypothesis, designed experiments, interpreted data, and polished the manuscript. H.Y.P., Y.M.Z., Y.J.L., L.X.F., and J.Z. performed in vitro and in vivo experiments; J.R.Z. and F.L. detected the clinical specimens; L.W. and N.Z. analyzed the data and performed the statistical analysis. All authors read and approved the final manuscript. Q.H.J., H.Y.Z., and Y.Z.L. directed and equally supervised the research.

COMPETING INTERESTS
The authors declare no competing interests.

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