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Clinical and biological impact of LINC02544 expression in breast cancer after neoadjuvant chemotherapy

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Abstract. – OBJECTIVE: Analysis of breast cancer and cancer tissue after neoadjuvant chemotherapy (nCT) may be helpful to find new biomarkers. It is known that long non-coding RNAs (lncRNAs) are involved in the carcinogenic pathway and drug resistance of breast cancer. Our aim was to determine the role of LINC02544 in the behavior and outcome of post-nCT breast cancer.

PATIENTS AND METHODS: The expression level of LINC02544 in breast cancer and its effect on the survival time of patients were predicted by lncAR database. *In vitro*, EdU, Wound-healing and transwell assays were used to detect the effects of LINC02544 on the proliferation, migration and invasion of MCF-7 cells, and the related regulatory networks were analyzed by the database. Quantitative Reverse Transcriptase-Polymerase Chain Reaction (QRT-PCR) was used to detect the expression of LINC02544 in 147 cases of nCT before and after treatment, and the relationship between the expression of LINC02544 and survival time and clinicopathological features was analyzed.

RESULTS: LINC02544 was highly expressed in breast cancer and led to poor prognosis. Overexpression of LINC02544 promoted proliferation, invasion and migration of breast cancer cells. Compared with the residual tumor after nCT with low expression of LINC02544, the high expression of LINC02544 in the residual tumor after nCT was significantly correlated with overall survival and disease-free survival.

CONCLUSIONS: In this study, it is suggested that LINC02544 has a potential application prospect as a biomarker and therapeutic target for breast cancer patients and neoadjuvant therapy patients.

Key Words:
Breast cancer, Neoadjuvant chemotherapy, LINC02544, Proliferation, Invasion.

Introduction

Breast cancer is the most severe malignant tumor both in China and around the world, accounting for the first cause of cancer-related mortality. In 2018, about 2.08 million new cases were confirmed worldwide and 626,679 people died of the disease[1]. Although with the development of medicine in recent years, considerable progress has been made in early diagnosis and systemic treatment of breast cancer patients, the recurrence and distant metastasis of breast cancer still hinder the cure of patients, which is an important reason for treatment failure. At present, almost all studies on breast cancer and tumors in other parts of the body are carried out from the point of view of tumor cell proliferation and metastasis. However, our understanding of the molecular mechanism of the occurrence and development of breast cancer is still limited to a large extent[2,3]. Therefore, it is necessary to better understand its molecular mechanism to find more specific and sensitive markers, accurately judge the prognosis of breast cancer, and give proper treatment for the final cure.

Fisher et al[4] proposed in the 1960s that breast cancer is a systemic disease. In the early stage of the disease, cancer cells can have micrometastasis to other places along with blood circulation and lymphatic circulation. Therefore, chemotherapy is a systemic treatment of breast cancer, the effect is also confirmed by a large number of clinical trials, and it can improve the survival rate and the quality of life of patients[5]. Then, in 1982, Frei et al[6] put forward the concept of neoadjuvant chemotherapy (nCT), which refers to receiving systemic chemotherapy before local treatment (surgery or plus radiotherapy), and continuing to complete the follow-up chemotherapy after local treatment. As a recognized and effective method for the treating of locally advanced and inflammatory breast cancer, nCT has been more and more popularized[7,8]. There are still some patients at risk of progression[9]. Therefore, there is

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an urgent need to select new sensitive biomarkers to determine which populations can benefit from neoadjuvant chemotherapy for breast cancer and which do not, so as to avoid the risk of disease progression.

LncRNAs play the role of oncogene or tumor suppressor gene in the occurrence and development of human cancer and can also be used as a key regulatory factor in the occurrence and development of tumor. Because of its frequent imbalance in cancer, it is of considerable significance to the diagnosis and prognosis of cancer, and is a potential tumor marker[10,11]. PCA3 (also known as DD3)[12] and prostate-specific transcript-1 (PCGEM1)[13] are the first abnormally expressed lncRNAs associated with cancer. At present, PCA3 is widely used as a biomarker of prostate cancer[14]. PCGEM1 is involved in the transcriptional activation of androgen receptors[15] and c-MYC activation, are both involved in the occurrence and development of prostate cancer[16]. The abnormal expression of MALAT1 (metastatic associated lung adenocarcinoma transcript 1) was first found in lung cancer as a prognostic indicator of survival in patients with lung cancer[17]. Now, MALAT1 has been found to be associated with many types of malignant tumors, including liver, breast, colon, prostate, stomach, brain, kidney and other organs[18], which also shows that it has a universal effect on cell proliferation. HULC was overexpressed in hepatoma cells[19]. The level of HULC transcription also increased in liver metastases of colon cancer. HOTAIR overexpression can also be used to predict liver cancer recurrence, with high expression in 65.7% of patients with liver cancer recurrence[20].

Uncontrolled cell proliferation is one of the known mechanisms for the occurrence and development of several cancers, and it is also an important feature of human cancer. A large number of studies have confirmed that lncRNA is involved in the regulation of cell proliferation in the process of tumorigenesis. For example, GAS5 is a tumor suppressor lncRNA and its disorders and gene mutations are closely related to breast cancer, prostate cancer, leukemia, gastric cancer and so on[21]. LncRNA HOTAIR is a necessary lncRNA, for breast cancer cell survival through estrogen binding to estrogen receptor (ER) and ER coregulators to regulate its transcription[22]. ANRIL is an antisense transcript of CDKN2B gene lncRNA which regulates its adjacent tumor suppressor CDKN2A/B, through epigenetic mechanism and then affects cell proliferation and apoptosis. ANRIL interacts with CBX7 (component of PRC1) and SUZ12 (component of PRC2) to induce gene silencing at INK4b-ARF-INK4a site[23], and then, regulates the role of CDKN2A/B. ANRIL can also play a role by inhibiting tumor suppressor p15. However, the research on the role of non-coding RNA in the development of breast cancer is not complete, and there are few studies in neoadjuvant chemotherapy.

In this study, we identified a new non-coding RNA LINC02544, overexpressed in breast cancer, experimentally explored the effect of LINC02544 on the malignant phenotype of breast cancer cells, and analyzed the relationship between the expression of LINC02544 in residual tumors after nCT treatment and the clinical outcome of the patients.

Patients and Methods

Clinical Patient Sample

The oncology department recruited 147 patients with invasive BC (stage II-III) who received continuous neoadjuvant chemotherapy (anthracycline and taxane) (Table III). The status of nodules before nCT was determined by ultrasound-guided fine needle puncture in the armpit and supraclavicular. Patients with negative lymph node metastasis underwent sentinel lymph node biopsy (SLNB) before chemotherapy. Whole-body bone scintigraphy and chest and abdomen CT were added to the staging workup to locally advanced tumors defined as cT3N1, CN2-3 or CT4. After chemotherapy and before operation, dynamic breast MRI was performed to determine the clinical response. Pathological complete response (pCR) is defined as ypT0/TisN0. This study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of China Medical University. All participates provided their written informed consent prior to study inclusion.

Cell Culture

MCF-7 cell lines were purchased from CHI Scientific, Inc (Wuxi, Jiangsu, China). The cells were cultured with complete medium including 89% 1640 and 10% FBS, both were purchased from Biological Industries (Beit-Haemek, Israel), and maintained in incubator with 37°C and 5% of CO_2 saturated humidity.

Clone Formation Experiment

The drug-treated cell suspension was inoculated into a Petri dish with 300 cells per dish. After the cells were evenly distributed, they were cultured in a cell incubator for 14-21 days. When a clone is visible to the naked eye, the culture is terminated. The medium was abandoned, and 4% paraformaldehyde was added to fix the cells for 15 min. After abandoning the fixed solution, Giemsa was added to dye for 15 min. Rinse with running water and dry. The number of effective cloned cells was observed under microscope.

qRT-PCR

RNA extraction was performed using TRIzol reagent. NanoDrop 8000 (Thermo Scientific, Waltham, MA, USA) was used to measure the concentration and purity of RNA. The single-stranded cDNAs were synthesized from 1 μg of RNA. The expression of mRNAs was quantified by RT-PCR with SYBR Green I (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences of genes were shown in Table I.

EdU Assay

Using EdU Cell Proliferation Kit (RiboBio, Guangzhou, Guangdong, China) to test proliferation. LECs were seeded in 24-well plates for transfection. Cells were added with 200 uL 50 uM EdU and incubated for 2 h. Apollo Dye Solution (red) were used to stain proliferating cells, nucleic acids in all cells were stained with 4',6-Diamidino-2-Phenylindole (DAPI) (blue) according to the protocols, and then, photographed.

Wound-Healing Assay

To test the cell migration ability of MCF-7, an in *vitro* wound healing assay was performed. Cells were seeded in 6-well plates until the cells formed a confluent monolayer, then, scratched using a 100 μL pipette tip. The scratch wounds were captured using phase-contrast microscopy at 0, 24 h. The relative wound size at each time point was analyzed by Image J.

Transfection

The MCF-7 cells were plated until the cell density reached 80% confluency of dishes to transfect. Plasmid of LINC02544 or small interfering RNA (si-RNA) of LINC02544 and were constructed by Genechem (Genechem, Shanghai, China). The plasmids were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Matrigel Transwell Assay

24-well Matrigel transwell (Corning, Corning, NY, USA) were used to investigated cell invasion. 2×10^5 MCF-7 were seeded on insert precoated with 1 μg/μL Matrigel (BD Biosciences, San Jose, CA, USA). Medium with FBS was used to stimulate invasion in the bottom of wells. After 48 h, the invasion cells were stained with 0.1% crystal violet.

Statistical Analysis

One sample Kolmogorov-Smirnov tests with Lilliefors significance correction were used to analyze normal distribution. Kaplan-Meier curves, log-rank test, Wilcoxon test and Cox proportional hazard regression multivariate models were used for disease-free survival (DFS), distant relapse-free survival (DRFS) and breast cancer-specific overall survival (OS) analyses. Kaplan-Meier curves validated by the log rank test. Data were shown as mean ± SD. Student's *t*-test or one-way ANOVA was used to compare the groups. Bonferroni-corrected for multiple comparisons. $p<0.05$ was considered significance.

Results

LINC02544 Is Highly Expressed in Breast Cancer and Is Associated with Poor Prognosis

First of all, we applied LnCAR (https://lncar.renlab.org/), a comprehensive open resources, to provide relevant public microarray data to comment the expression spectrum and prognosis of lncRNAs picture[24]. We found that LINC02544

Table I. Primer sequences.

Gene	Sequence (5'->3')	
LINC02544	Forward primer	TGTTCTCATTCGTGGCTGGA
	Reverse primer	GTCAGGCGGTTTCAGTGTTG
β-actin	Forward primer	CTACAATGAGCTGCGTGTGG
	Reverse primer	AAGGAAGGCTGGAAGAGTGC

was highly expressed in breast cancer (Figure 1A) and triple negative breast cancer (Figure 1C), and the high expression of LINC02544 was associated with low survival rate (Figure 1B, D).

Analysis of the Effects of LINC02544 on MCF-7 In Vitro

To verify the effect of LINC02544 on breast cancer cells, we constructed a LINC02544 plasmid and validated its overexpression efficiency (Figure 2A). The results showed that LINC02544 promoted proliferation (Figure 2B), invasion (Figure 2C) and migration (Figure 2D) of MCF-7 cells. Meanwhile, small interfering RNAs were constructed to silence the expression of LINC02544 (Figure 3A), and siRNA was found to inhibit proliferation (Figure 3B), invasion (Figure 3C) and migration (Figure 3D) of MCF-7 cells.

The Structure and Possible Regulatory Network of LINC02544

According to lnCAR database, LINC02544 is a full-length 544nt non-coding RNA, and there is only one transcript ENST00000564830. Its secondary structure prediction is shown in Figure 4A. The KEGG pathway in Figure 4B can further help us understanding its function. Figure 4C is the LINC02544 co-expression network. The detailed gene names and their correlation coefficients are shown in Table II. These data are helpful for us to further understand the mechanism of LINC02544.

LINC02544 Expression Associates with Poor Clinicopathological Breast Cancer

We examined the expression of LINC02544 in paraffin-embedded core biopsies of 147 breast cancer patients treated with nCT before and after treatment (Table III). The detection rate of pCR in the whole group was 18.4 %. During the last follow-up, 36 relapses and 28 deaths occurred after a median follow-up of 114 months for the entire group.

In BC biopsies before and after treatment, the expression of LINC02544 was relevant to invasive clinical features: grade 3 (Figure 5A) and triple negative phenotypes (Figure 5B). Extensive lymph node metastasis was also found in biopsies

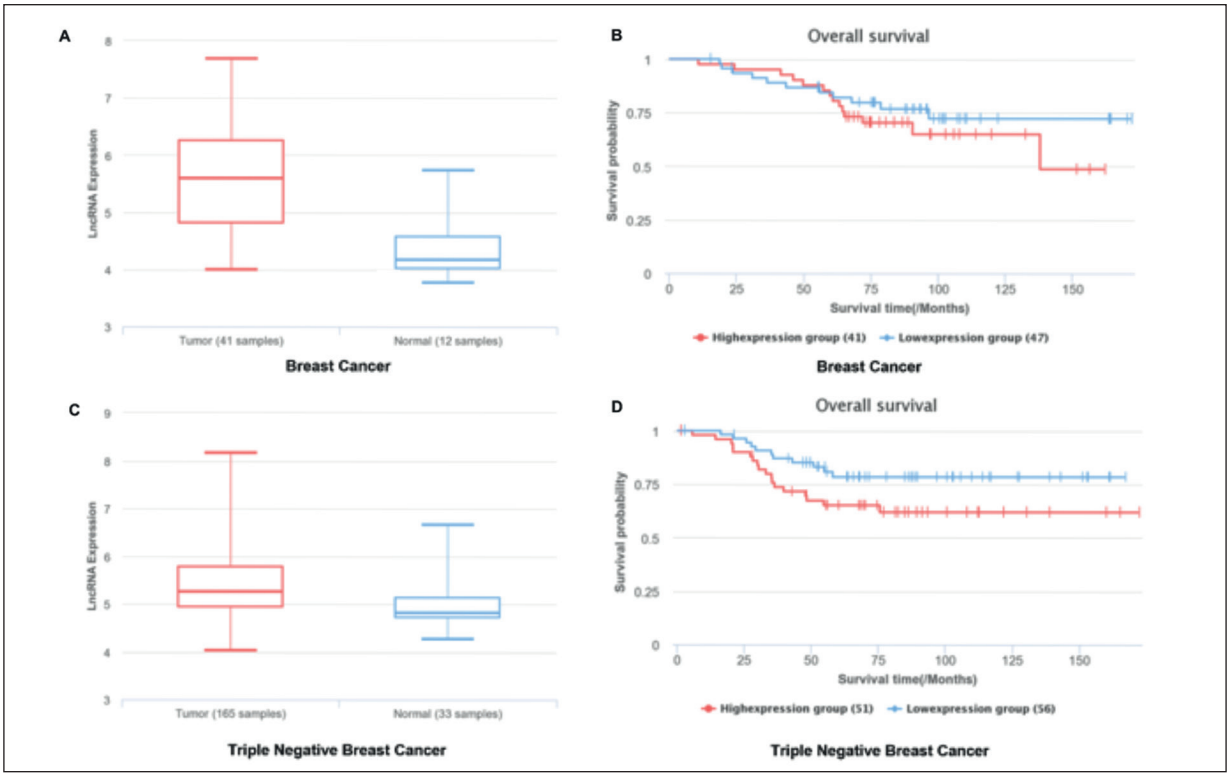

Figure 1. Expression of LINC02544 in breast cancer and its relationship with prognosis. **A**, According to the lnCAR database, LINC02544 is significantly highly expressed in breast cancer compared with normal tissue. **B**, Overall survival curve showed that patients with high LINC02544 expression had a lower survival rate. **C**, Significantly higher LINC02544 expression in triple negative breast cancer and (**D**) lower survival rates.

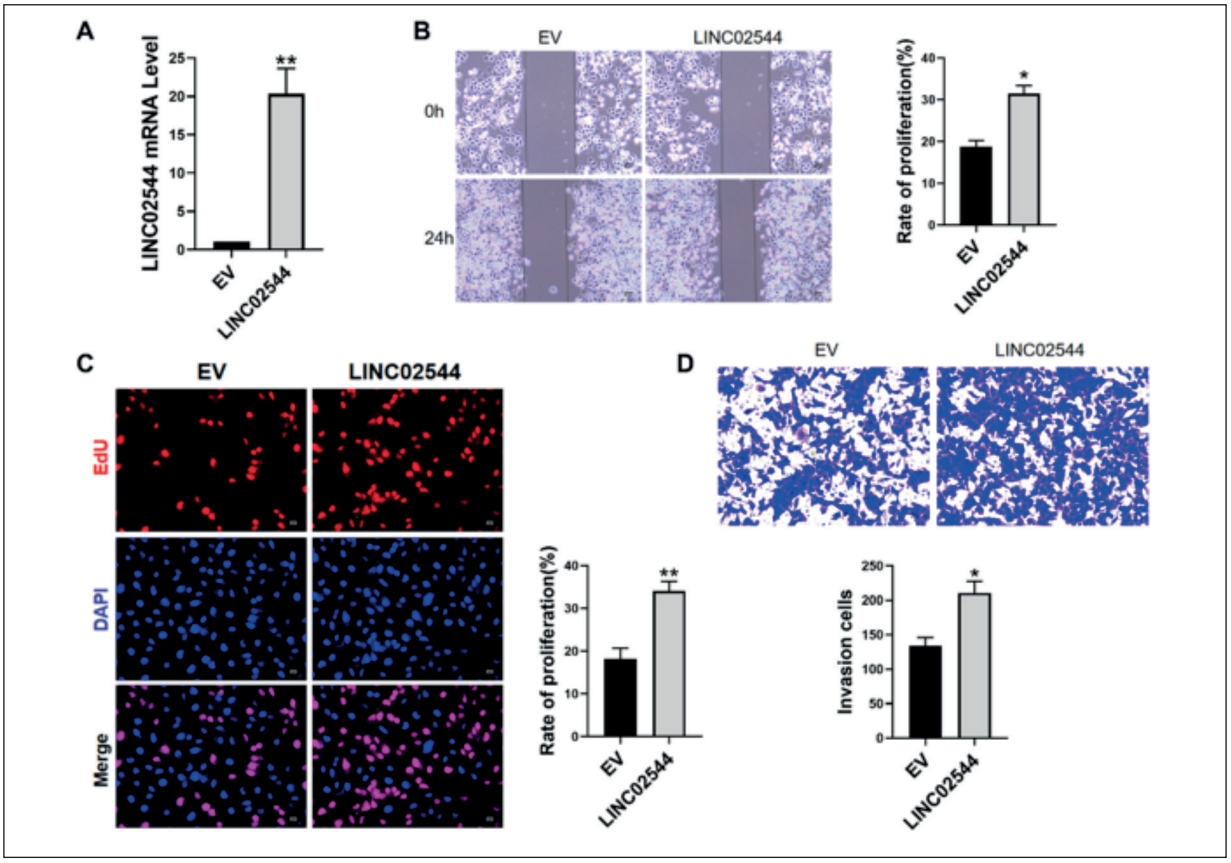

Figure 2. Overexpression of LINC02544 promoted MCF-7 proliferation phenotype. **A,** qRT-PCR was used to confirmed mRNA expression of LINC02544 to detect the overexpression efficiency. **B,** Wound healing tested revealed that overexpression of LINC02544 increased cell migration, (magnification, ×40). Analysis of Wound healing assay. **C,** EdU staining illustrated that forced expression of LINC02544 promoted cell proliferation, (magnification, ×200). Analysis of EdU staining, n=6. **D,** Transwell assay showed invasion ability of MCF-7 was promoted by LINC02544, (magnification, ×100). Analysis of transwell assay. *$p < 0.05$; **$p < 0.01$.

after chemotherapy, but there was no significant change in pretreated samples (Figure 5C). There was no correlation between baseline LINC02544 expression and tumor size. After nCT treatment, the expression of LINC02544 continued to increase (Figure 5D). No correlation was observed between LINC02544 expression and Polymerase Chain Reaction before nCT treatment (Figure 5E, F), indicating that the level of LINC02544 was not related to chemotherapy resistance. The expression of LINC02544 was measured by immunohistochemical surrogate subtypes, it showed that the expression of Pre-nCT in triple negative tumors was significantly higher than that in HR+HER2- tumors.

Effect of High LINC02544 Expression on BC Survival Rate After Chemotherapy

We analyzed whether the high expression of LINC02544 in residual breast tumors after nCT was related to the prognosis of patients. The median of LINC02544 expression after nCT was arbitrarily pre-defined as the cut-off point between the two groups: LINC02544 high expression group and LINC02544 low expression group. In univariate analysis (Figure 6A), we found that the high expression of LINC02544 in residual tumors of patients without Polymerase Chain Reaction predicted a significant deterioration of OS, DFS and DRFS. The prognostic effect of LINC02544 expression in post-chemotherapy residual tumors was confirmed in a multivariate Cox proportional hazard regression model, which also included other clinical and pathological variables that were significant or almost significant in previous univariate analysis: tumor subtypes (TNBC and others), posterior nCT lymph node involvement (ypN+ *vs.* ypN0), and DFS grade 3 (*vs.* 1-2) (Table IV). For DRFS and OS, multivariate models, only LINC02544 expression, lymph node metastasis

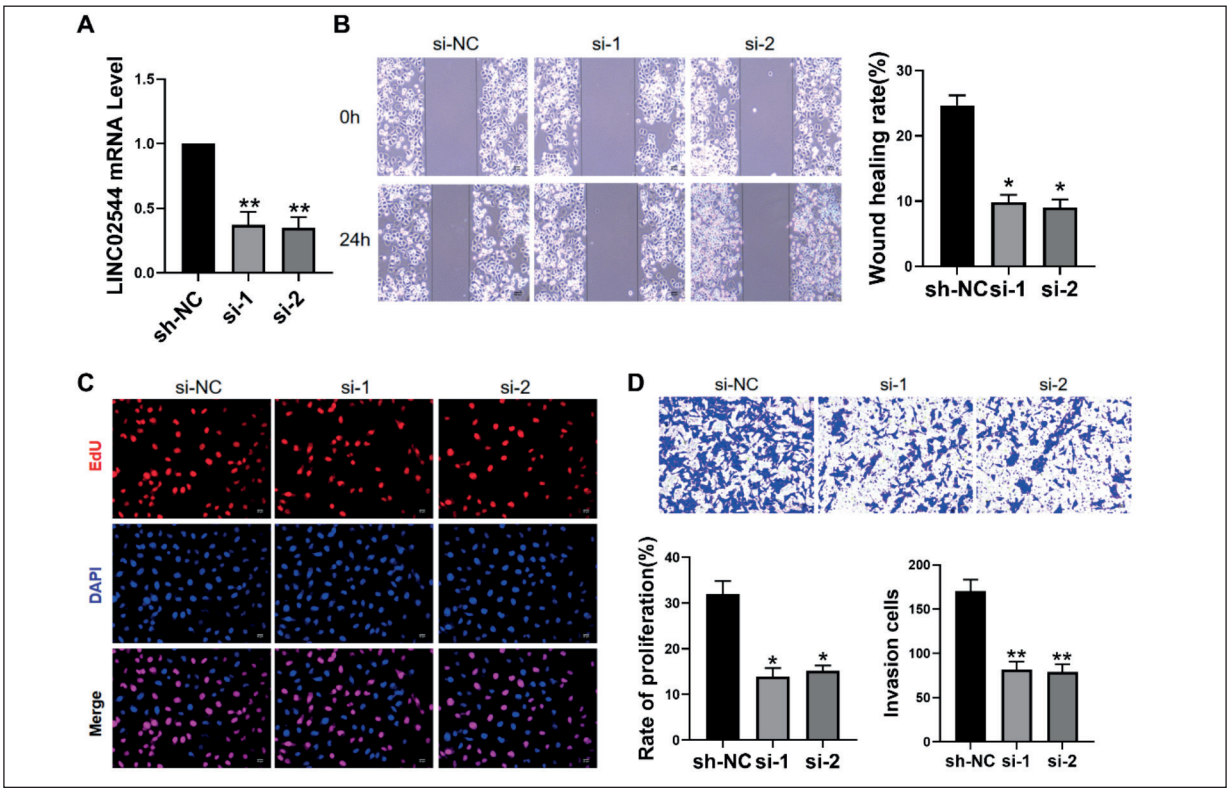

Figure 3. Inhibition of LINC02544 ameliorated MCF-7 proliferation invasion and migration. **A,** qRT-PCR was used to detect mRNA expression of LINC02544 to detect the efficiency of si-RNA. **B,** Wound healing tested revealed that inhibition of si-RNA ablated the cell migration, (magnification, ×40). Analysis of Wound healing assay. **C,** EdU staining illustrated that knockdown of LINC02544 inhibited cell proliferation, (magnification, ×200). Analysis of EdU staining. n=6. **D,** Transwell assay showed invasion ability of MCF-7 was inhibited by si-RNA, (magnification, ×100). Analysis of transwell assay. *$p < 0.05$; **$p < 0.01$.

after nCT and grade 3 were included. By doing so, the high expression of LINC02544 in residual tumors was found to be a significant marker of the deterioration of OS, while no significant trend of DFS and DRFS was found (Table IV). Survival analysis according to post-nCT subtypes showed

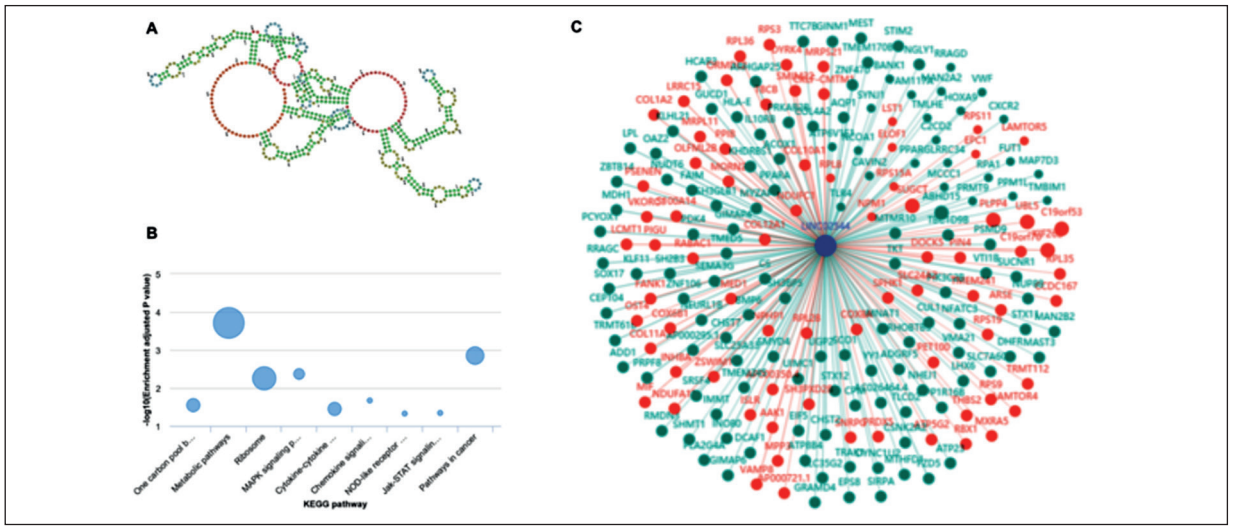

Figure 4. Analysis of LINC02544 regulatory network. **A,** LINC02544 secondary structure. **B,** Analysis of B LINC02544 associated KEGG pathways. **C**, LINC02544 correlation co-expression network diagram.

Table II. Primer sequences.

LINC02544	1	LINC02544	1	LINC02544	1	LINC02544	1	LINC02544	1
PLPP4	0.7329	GPM6B	-0.529	CILP	0.4928	COL1A1	0.4696	PDZK1IP1	-0.4536
LAMP5	0.7028	NFIB	-0.5276	GPR68	0.4927	KLHL35	0.4684	BNC2	0.4527
LRRC15	0.6467	SEPT10	-0.5272	CEMIP	0.4925	SAV1	-0.4682	DHRSX	0.4524
COL10A1	0.6447	SERPINB5	-0.5272	CRYAB	-0.4917	ENC1	0.4681	CNTNAP3B	-0.4522
COMP	0.6338	GPRC5B	-0.526	ADAMTS6	0.4903	PNRC1	-0.4677	AEBP1	0.4522
SYNDIG1	0.6202	NOX4	0.5248	KRT5	-0.4901	TBC1D30	0.4668	ARX	0.4519
SLC4A8	0.615	CEP290	0.5234	CENPP	0.4867	CROT	0.4666	TNFSF4	0.4519
RAB26	0.6075	RGMA	-0.5229	NOB1	-0.4866	KIT	-0.4666	C3orf58	-0.4518
COL11A1	0.5913	PPARA	-0.5229	MOB3B	-0.4859	FABP7	-0.4664	TCF7L2	-0.4517
SFRP1	-0.5869	ST6GAL2	0.5219	MICAL2	0.4854	FDXACB1	0.4662	PDLIM3	-0.4516
P4HA3	0.5773	HTR2B	0.5216	FN1	0.4849	RCAN1	-0.4662	PKIA	0.4515
MYC	-0.5653	ITGA6	-0.5211	ZNF385A	0.4839	ROGDI	0.4658	BRAP	0.4515
CGA	0.5577	CDH11	0.5188	SPOCK1	0.4837	RPS25	-0.4649	PLSCR1	-0.4513
ROPN1B	-0.5569	POLR3B	0.5188	ZNF467	0.4832	SYNM	-0.4644	IGSF5	0.4512
CNBP	-0.5567	SYT17	0.5156	NAB1	-0.483	ZNF644	-0.4642	VCAN	0.4511
COL5A2	0.555	SOSTDC1	-0.5145	PCDH7	0.4829	MRAS	-0.4642	DDX18	-0.4507
TMEM43	-0.555	COL1A2	0.5141	FAP	0.4829	ZNF468	0.4641	SULT1A3	0.4492
WISP1	0.554	ZNF418	0.5135	TM4SF1	-0.4826	TMEM220	-0.4638	SULT1A4	0.4492
TMTC3	0.5524	HOXC4	0.5132	SLPI	-0.4823	COBL	-0.4631	BCAS4	0.4489
NDRG2	-0.5512	KPTN	0.5125	STAC2	-0.482	RGS22	0.4623	WIF1	-0.4487
VSTM2A	0.5509	COL5A1	0.5114	SPOP	0.4816	MAML2	-0.4617	HPS5	-0.4486
ADAMTS12	0.5495	C1QTNF5	0.5103	SMIM22	0.4813	PRR15	0.4616	VPS41	0.4477
HOXC9	0.5483	MFRP	0.5103	PRNP	-0.4812	CCNL1	-0.4613	ZBTB37	0.4476
ATP7B	0.548	RUNX2	0.509	PARG	0.4809	SULT1A2	0.461	CACFD1	0.4476
PTK6	0.5463	ADGRV1	0.5088	SYT13	0.4805	TF	-0.4609	CACFD1	0.4476
PRDM6	0.5429	C10orf82	0.5072	SLX4IP	0.4797	CACNG1	0.4584	NUDCD3	0.4476
CILP2	0.5428	GCHFR	0.5063	CHI3L1	-0.4796	SCPEP1	-0.4584	SPRY2	-0.4475
PVALB	0.541	ADD3	-0.5056	ID4	-0.4795	MBNL1	-0.4581	RABEPK	0.4475
C1QTNF3	0.5383	SLC24A2	0.5045	CMTM7	-0.4793	KIFC2	0.4576	KLHL13	-0.4473
SOX10	-0.5381	TANK	-0.5034	EME1	0.4758	KIAA0391	0.4575	GSTP1	-0.4473
MMP11	0.536	INHBA	0.5032	EPN3	0.4757	DNASE1	0.4572	KLF11	-0.4471
HSD17B6	0.5354	SALL4	0.5024	PELI1	-0.4755	ROPN1	-0.457	OPN1LW	-0.4459
CEP83	0.5342	THY1	0.5022	CDH3	-0.4752	GRAMD2B	-0.4569	OPN1MW	-0.4459
NTM	0.5337	C12orf73	0.4984	ARRDC3	-0.4749	MFGE8	-0.4569	OPN1MW2	-0.4459
SOX9	-0.5334	PTPRD	0.4974	KRT17	-0.473	AMACR	0.4567	OPN1MW3	-0.4459
CX3CL1	-0.533	RABEP2	0.4974	ANKRD50	0.4718	DMD	-0.4564	APOC1	0.4456
DOLPP1	0.5325	NUAK1	0.4971	MXI1	-0.471	TACSTD2	-0.4563	NAA60	0.4454
ANAPC7	0.5304	TPRN	0.4956	SULF1	0.4708	CCDC125	0.4562	POTEM	0.4454
TRIM29	-0.5302	CERCAM	0.494	MMP13	0.4707	BICDL1	0.4543	SMARCA2	-0.4443
PTPDC1	0.5295	BBOX1	-0.4928	HOXC11	0.4696	CORO2A	0.4539	BOC	-0.4439

that the effect of high expression of LINC02544 on prognosis mainly occurred in HR+tumors (Figure 6B), but it was not statistically significant, and the effect was more significant in early follow-up. According to the clinical definition of endocrine drug resistance, exploratory comparison of recurrence types showed that 9/10 recurrence met the primary or secondary endocrine drug resistance criteria in LINC02544 high expression group, while only 2/6 recurrence occurred in LINC02544 low expression group. There was no significant difference between the expression of LINC02544 and prognosis in HR tumors after nCT. Compared with the results after chemotherapy, there was no correlation between preconditioning expression of LINC02544 and different prognosis: there was no significant difference in survival rate between patients with low and high levels of preconditioning LINC02544 (Figure 6C).

MCF-7 Transfected with LINC02544 Showed In Vitro Resistance to Tamoxifen and Was Rich in Endocrine Resistance Signals

To identify the potential therapeutic role of LINC02544 expression in BC, we examined the effects of tamoxifen alone or in combination with everolimus or Palbociclib on estradiol supplementing (10 nM) MCF-7 cells transfected with LINC02544. Compared with control, cells

Table III. Patient characteristics.

Characteristic	N	%	Characteristic	N	%
N	147	100%	**Prechemotherapy IHC subtypes**		
Age (median; min-max)	54.4 (20-78)		HR+ HER2-	51	34.7
Menopausal status			HR+ HER2+	26	17.7
Postmenopausal	76	51.7	HER2+ HR-	35	23.8
Premenopausal	71	48.2	TNBC	32	21.8
Clinical stage			Not available	3	2.0
IIA	26	17.7	**Treatment scheme**		
IIB	39	26.5	ACx4 - Docetaxelx4	104	70.7
IIIA	47	31.9	Anthracyclines & weekly paclitaxel	22	15.0
IIIB	9	6.2	Scheme with anthracyclines & concomitant taxanes	12	8.2
IIIC	26	17.7	Other schemes	9	6.1
Clinical stage of primary tumor			**Treatment with trastuzumab**		
cT1-2	68	46.3	No	16	78.9
cT3- 4	79	53.7	Neoadjuvant and adjuvant	23	15.6
Lymph node clinical stage			Only adjuvant	8	5.4
cN0- -1	79	53.7	**Pathologic complete response** (pCR) (n= 119)		
cN2- -3	68	46.3	pCR	22	18.4%
Histological type			No pCR	97	82.4%
Ductal	137	93.2	**Posttreatment tumor stage in patients without pCR** (n= 97)		
Lobular	6	4.1	ypT0-Tis	5	5.1
Others	4	2.7	ypTlmic	4	4.1
Histological tumor grade			ypTla-c	21	21.6
GI-II	56	38.1	ypT2	42	43.3
GIII	77	52.4	ypT3	20	20.6
Not available	14	9.5	ypT4	5	5.1
Lymphovascular invasion			**Posttreatment lymph node stage in patients without pCR** (n= 97)		
No	102	69.4	ypN0	25	25.8
Yes	36	24.5	ypN1mic	6	6.2
Not available	9	6.1	ypN1	23	23.7
			ypN2	21	21.6
			ypN3	7	7.2
			N/A*	15	15.5

*N/A corresponds to patients with pretreatment sentinel node biopsy with pNO (sn) result, in whom a post treatment lymphadenectomy was not performed.

transfected with LINC02544 did not slow their growth exposed to tamoxifen, but the resistance was reversed by the combination of tamoxifen with everolimus or Papoxilie, showing a similar response pattern to control cells (Figure 7A, B).

Discussion

Breast cancer is one of the deadliest female malignant tumors in the world, and the incidence is getting younger. Data released by the World Health Organization in recent years show that the global mortality rate of female breast cancer is about 13.7%[25]. The diagnosis and treatment of breast cancer have entered the molecular level. According to the molecular classification of breast cancer, we can better carry out individual and comprehensive treatment for breast cancer patients, thus prolonging the overall survival time (OS, overall survival) of these patients. Therefore, although the incidence of breast cancer morbidity is increasing, the mortality rate of breast cancer is decreasing, thanks to the systemic and accurate comprehensive treatment of breast cancer. In recent years, cancer research centers in many countries and regions around the world have conducted a large number of studies on breast cancer neoadjuvant chemotherapy (nCT), breast cancer neoadjuvant chemotherapy research, including many adjuvant chemotherapy programs.

Here, we identified that the high expression of LINC02544 may be a sign of poor OS and DFS after nCT. *In vitro* data show that this

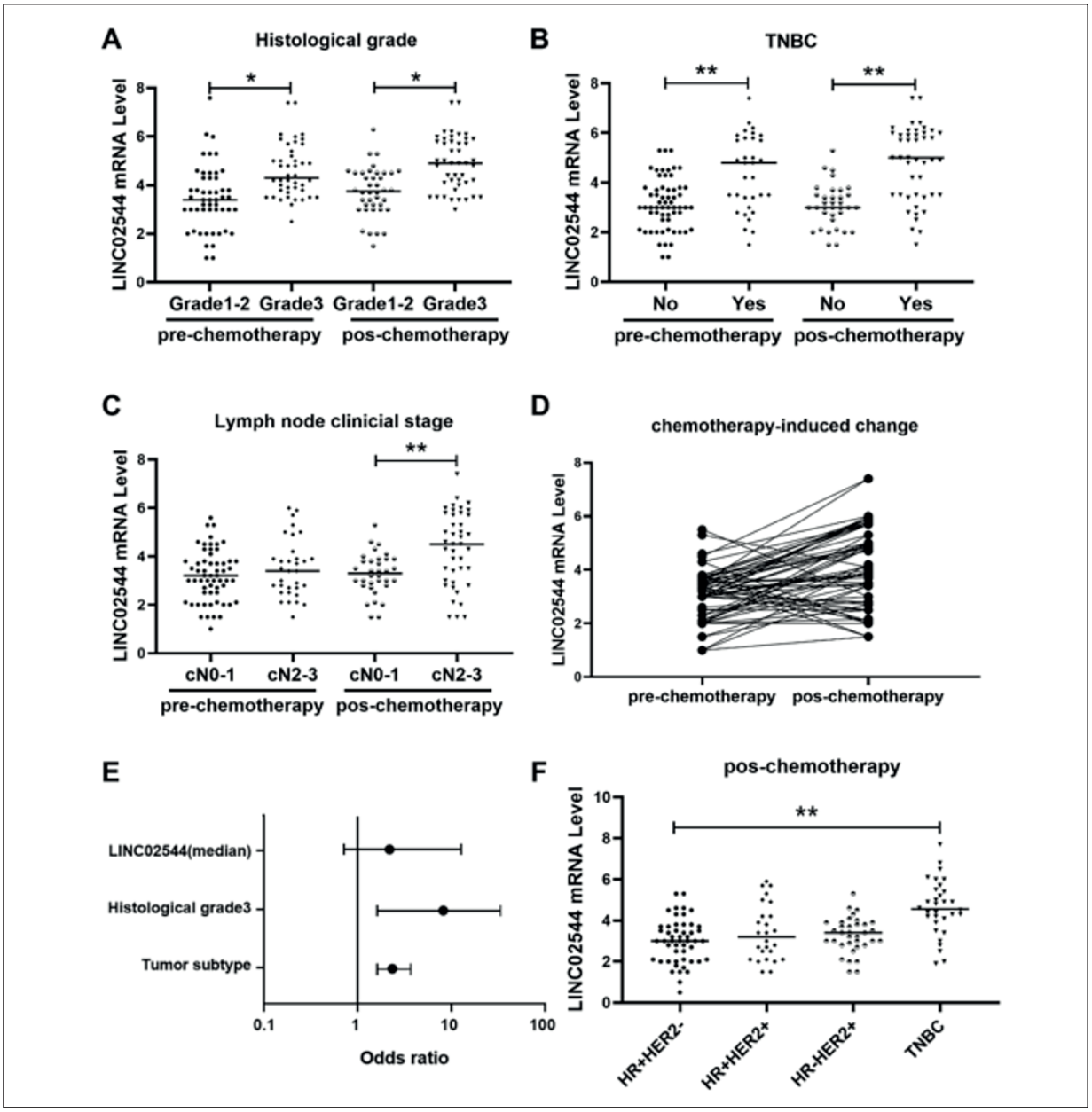

Figure 5. Relationship between clinicopathological features and LINC02544 expression before and after neoadjuvant chemotherapy. **A,** Patients with poorly differentiated tumors (51 patients with grade 1-2 before CT; 44 patients with grade 3 before CT; 40 patients with grade 1-2 after CT; 45 patients with grade 3 after CT). **B,** Triple negative phenotype (61 cases no TNBC before CT; 33 cases of TNBC before CT; 35 cases no TNBC cases after CT; 45 cases of TNBC after CT. **C,** Extensive (CN2-3) lymph node metastasis (cN0-1 before CT, n=59; cN2-3 before CT, n=33; cN0-1 after CT n=34; cN2-3 after CT, n=43). **D,** Chemotherapy induced increased LINC02544 expression (n=66). **E,** LINC02544 level was not associated with pathological (OR=3.296;95 % CI = 0.672-7.197; $p = 0.201$). **F,** Different breast cancer subtypes before chemotherapy (HR+HER2-, n=51; HR + HER2 +, n = 26; HR - HER2 +, n = 35; TNBC, n=32).

prognostic impairment may be related to the involvement of LINC02544 in the proliferation and invasion of BC cells, suggesting the potential use of LINC02544 as a prognostic marker. According to clinical sample study, the expression of LINC02544 in residual tumors after chemotherapy may represent the drug resistance part of the primary tumor and is related to the proliferation. Through KEGG analysis, we found that LINC02544 is related to many signal pathways.

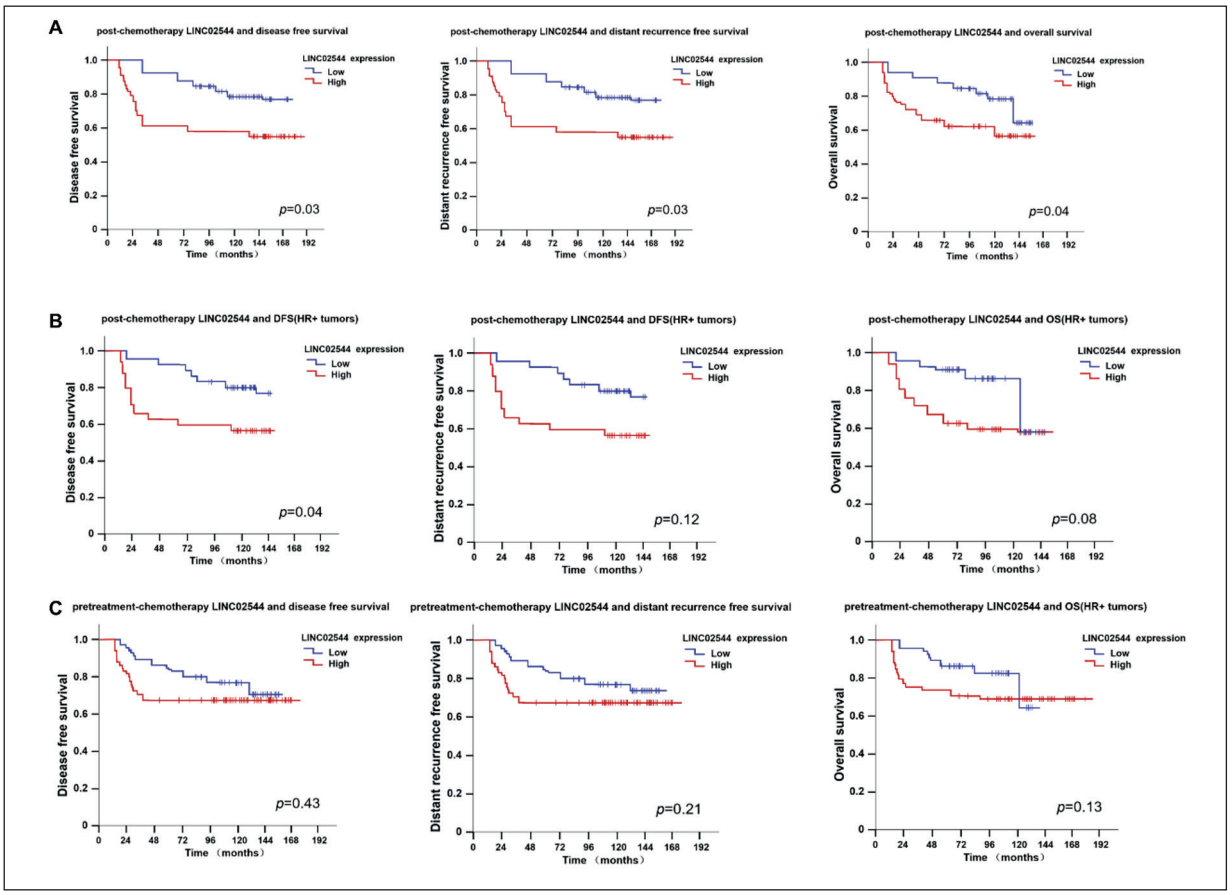

Figure 6. Survival analysis based on low or high expression of LINC02544. **A,** Kaplan-Meier survival curves of DFS, DRFS and OS were plotted according to the level of LINC02544 in patients with residual breast tumor (n=80) after chemotherapy (with the median after nCT as the cut-off point, high and low). **B-C,** Kaplan-Meier survival curves for DFS, DRFS and OS after chemotherapy (after nCT).

The correlation analysis of LINC02544 co-expression genes showed that these genes, such as LAMP5, LRRC15 and COL10A1, were associated with the poor prognosis (low survival rate) marker of cancer[26-28], but we did not carry out further experimental verification.

Table IV. Univariate and multivariate survival analysis of patients with post-nCT breast residual tumors according to LINC02544 expression (high *vs*. low)(n = 80).

Variables*		Univariate analysis HR (95% CI)	*p*	Multivariate analysis HR (95% CI)	*p*
DFS	LINC02544	2.54 (1.09- -5.96)	0.03	2.44 (0.99- -6.02)	0.053
	ypN+	6.37 (2.22- -18.28)	< 0.001	7.10 (2.06- -24.41)	0.002
	Grade 3	2.23 (1.03- 4.83)	0.04	1.83 (0.71-4.71)	0.21
	TNBC	2.14 (0.98- -4.68)	0.056	2.91 (1.16 -7.30)	0.02
DRFS	LINC02544	2.33 (0.98- -5.51)	0.053	2.44 (0.98- -6.04)	0.054
	ypN+	13.08 (3.10- -55.22)	< 0.001	9.05 (2.10- -39.00)	0.003
	Grade 3	1.92 (0.87- 4.23)	0.11	2.15 (0.87- -5.34)	0.10
OS	LINC02544	2.72 (1.05-7.05)	0.039	2.90 (1.02-7.78)	0.046
	ypN+	9.71 (2.28- 41.39)	0.002	7.31 (1.69- -31.73)	0.008
	Grade 3	2.32 (0.98- -5.48)	0.054	2.35 (0.90- -6.16)	0.08

*Variables: LINC02544 post-nCT high expression (over the median), ypN+ (post-nCT nodal involvement), histological grade 3 (*vs*. grade 1-2), TNBC(triple negative breast cancer vs. other breast cancer subtypes).

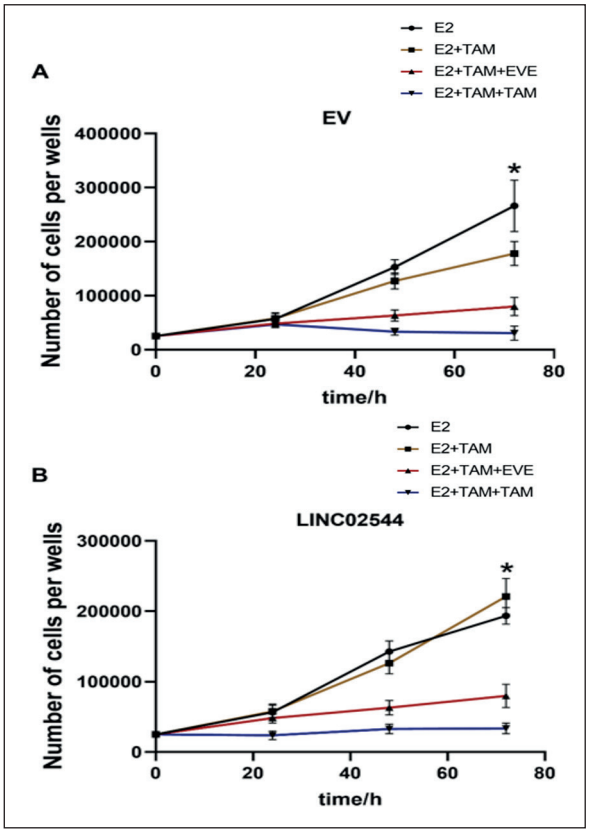

Figure 7. Sensitivity to hormonal therapies in MCF-7 cells transfected with LINC02544. **A,** MCF-7 cells or (**B**) transfected with LINC02544 were stimulated with estradiol (E2, 10 nM) and treated with TAM, tamoxifen plus everolimus (EVE) or tamoxifen plus paloxib (PAL). The cells were tested 72 hours later.

In the neoadjuvant therapy environment, new biomarkers are needed to guide treatment strategies. Therefore, the characteristics of LINC02544 as a prognostic biomarker related to high proliferation and endocrine drug resistance, if further confirmed, may be helpful to identify high-risk BC patients with residual diseases after nCT. In addition to patients choosing new adjuvant strategies, LINC02544 may also be a target for invasive tumor therapy.

Our current work has some limitations that should be taken into account. First of all, the significant prognostic correlation of LINC02544 in tumors may be related to relatively small sample size. Therefore, our results support the effect of LINC02544 on the prognosis of HR-positive tumors, but it may not be sufficient to rule out similar effects of HR-negative BC. Secondly, while our experimental results seem to be consistent with our clinical and pathological findings, further understanding that LINC02544 may play a role by activating or silencing downstream factors, a more comprehensive analysis of LINC02544 and endocrine drug resistance, or a more accurate classification of tumors according to molecular subtypes. In the future research, we need to further clarify the mechanism of LINC02544 involved in breast cancer.

Our results show that LINC02544 can be used as a new prognostic factor for residual tumors after nCT, and it is closely related to high proliferation, high invasion and migration of breast cancer cells. At present, there are few studies on the role of lncRNA expression in the occurrence and development of breast cancer, let alone the relationship between LINC02544 and the efficacy of neoadjuvant chemotherapy for breast cancer. This paper studies the relationship between LINC02544 and the efficacy of neoadjuvant chemotherapy for the first time, hoping that LINC02544 can become another powerful predictor of chemotherapy efficacy, so as to serve the clinic. This research has achieved preliminary results. In the future, we will continue to expand the number of cases, prolong the observation time for further investigation, and make a comprehensive analysis combined with multiple indicators and factors in order to more accurately predict the effect of chemotherapy, further understanding the biological mechanism behind these findings, and further verifying it in a larger clinical size, in order to clearly establish the correlation and effectiveness of LINC02544 in breast cancer.

Conclusions

To summarize, bioinformatics analysis, *in vitro* and clinical data support the role of LINC02544 in breast cancer cell proliferation and endocrine drug resistance suggests its potential use as a biomarker for breast cancer patients and neoadjuvant therapy patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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