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LncRNA LINC01116 competes with miR-145 for the regulation of ESR1 expression in breast cancer

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Abstract. – OBJECTIVE: To investigate the biological role and clinical significance of long non-coding RNAs (lncRNA) LINC01116 in breast cancer.

MATERIALS AND METHODS: In the public database Gene Expression Omnibus (GEO), the breast cancer data set GSE54002 was screened for differentially expressed lncRNA LINC01116 in breast cancer tissues and paracancerous tissues. Quantitative Real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of LINC01116 in 64 breast cancer tissues and 30 normal breast tissues. Level of LINC01116 and clinicopathological parameters of breast cancer were statistically analyzed. The effect of LINC01116 in breast cancer cells was investigated after knockdown of LINC01116. Luciferase reporter gene was further used to investigate the mechanism of endogenous RNA (ceRNA).

RESULTS: Results of GSE54002 showed that the expression of LINC01116 in breast cancer tissues was significantly increased. In clinical samples, the level of LINC01116 in patients with breast cancer was significantly increased, which was correlated with the overall tumor size and tumor node metastasis (TNM) stage in patients, but not correlated with the age, sex and lymph node metastasis ($p>0.05$). LINC01116 act as an endogenous sponge and directly bind miR-145, resulting in the upregulation of estrogen receptor 1 (ESR1), a target gene of miR-145.

CONCLUSIONS: LncRNA LINC01116 is highly expressed in breast cancer and is a new prognostic biomarker in breast cancer. Our study establishes a new link between LINC01116, miR-145 and ESR1.

Key Words

LncRNA, Breast cancer, ceRNA, LINC01116, miR-145, ESR1.

Introduction

Breast cancer is the most common malignant tumor in females. Through the overexpression of proto-oncogene accompanied by the inhibition of tumor suppressor gene, breast cancer escapes the immune destruction and achieves uncontrolled proliferation[1]. Gene therapy has become a popular cancer treatment to achieve permanently correct abnormal gene expression associated with the development of breast cancer[2].

A great number of investigations have demonstrated that microRNAs (miRNAs), messenger RNA (mRNA), pseudogenes, long non-coding RNAs (lncRNAs), and circular RNAs form complex networks can regulate expressions of each other[3]. Poliseno et al[4] proposed a competitive endogenous RNA (ceRNA) hypothesis that endogenous RNAs such as mRNA, pseudogene, lncRNA, etc., contained certain miRNA binding sites through which competitive binding of the shared miRNAs can reduce the miRNA suppression of targeting mRNA, thus improving expressions of target genes. A series of biological behavior was regulated, including the occurrence of tumors[5,6]. More and more evidence has shown that ceRNA was closely related to tumorigenesis. LncRNAs are non-coding RNAs with over 200 nucleotides in length and are involved in many disease processes. In recent years, studies have shown that lncRNA can be used as a ceRNA, which is closely related to the development of breast cancer. The driving genes compete for the miRNA response elements, impair the inhibitory effect of miRNA on the target genes, and indirectly regulate the target gene expression level, thereby participating in the regulation of breast cancer progression. LncRNA LINC01116, also known as TALNEC2, is located in the 2q31.1 region. LINC01116 has been reported on the occurrence and development of tumors[7]. To date, no research has been done on the relationship between LINC01116 expression and the incidence and prognosis of breast cancer. In this work, we found that LINC01116 was overexpressed in breast cancer, which was associated with metastasis of breast cancer and poor prognosis. Fundamental mechanism analysis revealed that LINC01116 can act as a ceRNA to regulate estrogen receptor 1 (ESR1) expression by competing with miR-145.

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Materials and Methods

Cell Culture

Cells were seeded in culture flasks in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and cultured at 37°C in a cell incubator with 5% CO_2.

Cell Transfection

Cells were seeded in the 6-well plate. When the cell density was about 70%, cells were transfected with si-NC and si-LINC01116 sequences according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection kit, and the medium was changed 6 h after transfection. Interference sequences were as follows: si-LINC01116 1 #: CCAAAGGCCCTGAAGTACACAGTTT; si-LINC01116 2 # AGCAGTGTATTAGAAGACAACTGAA.

RNA Extraction and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

24 hours after transfection, the cells were resuspended with 1 mL of TRIzol and the total cell RNA was extracted and cDNA was reverse transcribed following instruction. The expression level of LINC01116 was detected by qRT-PCR using a 10 µL system. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as internal control and each sample was performed in triplicate. Primer sequences were as follows: LINC01116 (Forward) 5'-GTTCAAGTGCCCGGGTTT-3', LINC01116 (Reverse) 5'-CTTTCAGGCGG-3'; GAPDH (Forward) AGCGAGATCCCGCCAACA, GAPDH (Reverse) GGCCGTCACGCCACATC

Cell Proliferation Assay by Cell Counting Kit-8 (CCK8) Method

Two groups of transfected HCC cells were seeded in a 96-well plate, the serum-free medium was replaced after cells were cultured for 24, 48, 72 and 96 h. 10 µL of CCK8 were added to each well and incubated at 37°C and 5% CO_2 for 1 h. Finally, OD value was measured at 450 nm. Each experiment was performed in quintuplicate.

Cloning Formation Assay

Two HCC38 cells transfected for 24 h were inoculated into the medium plate at a density of 3×10^3/100 µL and maintained for 24 h in an incubator at 37°C, 5% CO_2, medium replaced every 2 d and the culture was terminated after 14 d. Medium was then removed and cells were washed with the phosphate buffered saline (PBS) twice, fixed with 5% paraformaldehyde for 30 min. After fixation, remaining liquid was removed and 1 mL of 0.1% crystal violet solution was added per well for 30 min incubation. Then, excessive crystal violet solution was removed, and cells were washed until the solution was clear with PBS. Visible colonies were counted and recorded.

Luciferase Reporter Gene

Cells were seeded in a 24-well plate, and each sample was performed in triplicate. Cells were transfected when the confluency was 80% to 90%. Specifically, cells were transfected with 80 ng of plasmid, 5 ng of Renilla luciferase, 50 nM of miR-145 mimics and a negative control using Lipofectamine 2000. After 24 h transfection, cells were collected and measured. The corrected enzyme activity per sample well was calculated as follows: Luciferase activity value/Renilla luciferase activity value.

Statistical Analysis

Statistical product and service solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis, and GraphPad Prism 5.0 (Version X; La Jolla, CA, USA) was used for picture editing. Measurement data were compared with *t*-test and presented as mean ± standard deviation ($\bar{x} \pm s$). Categorical data were compared with x^2-test. $p<0.05$ indicated statistically significant difference; $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

Results

Relationship Between the Expression of LINC01116 in Patients with Breast Cancer and Clinical Data

GSE54002 data analysis showed that LINC01116 expression was significantly increased in breast cancer (Figure 1B, $p<0.001$). Further qRT-PCR analysis in 64 breast cancer patients and 30 normal breast tissues confirmed that LINC01116 was highly expressed in breast cancer tissues (Figure 2A, $p<0.001$). The clinical data of patients were also analyzed. The overall survival rate of LINC01116 overexpression group was lower than that of the low expression group

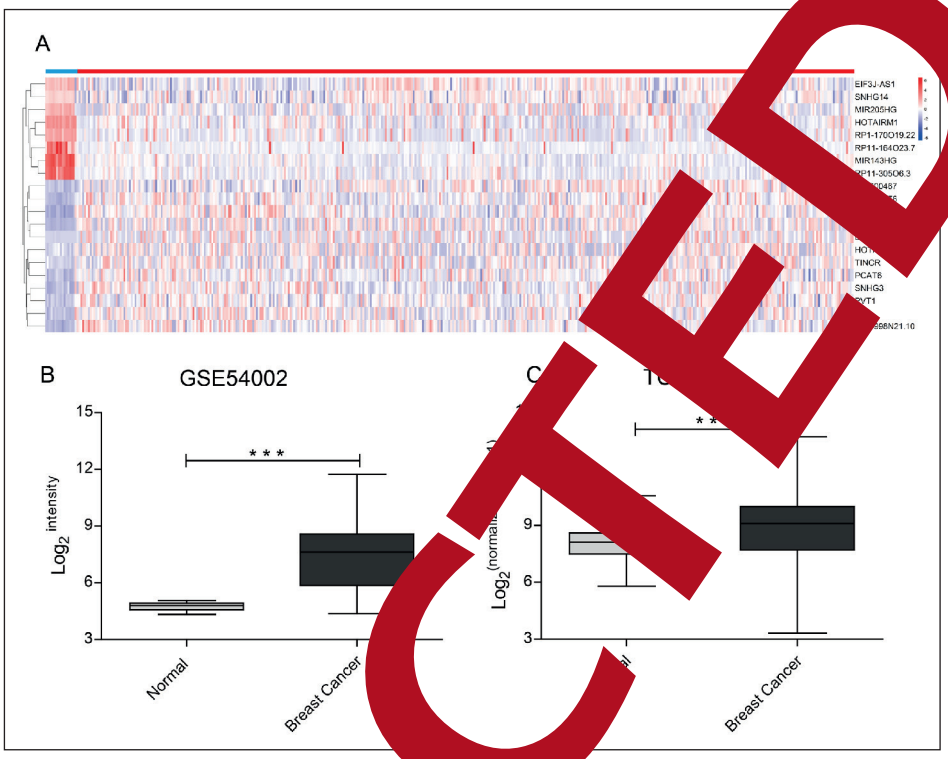

Figure 1. Long non-coding RNA LINC01116 is highly expressed in breast cancer tissues. **A**, Heat Map of differential expression between breast cancer tissues and normal breast tissues. **B**, Analysis in GSE54002 dataset shows that LINC01116 is highly expressed in breast cancer tissues.

(Figure 2B, p=0.0283 HR=1.801). In addi… 2D, p<0.001). x^2-test results showed that in the expression of LINC01116 was higher … high expression group, the advanced tients with advanced tumors and tumors … was correlated with larger tumor larger volume (Figure 2C, p<0.001 and Fig… volume (Table I).

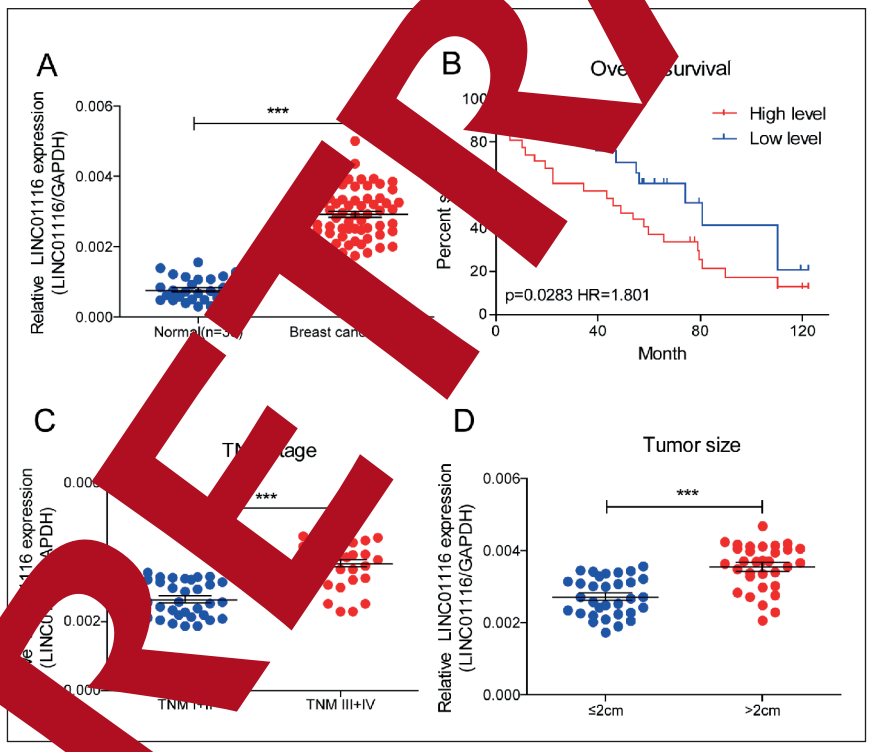

Figure 2. LINC01116 promotes breast cancer tissue proliferation. **A**, LINC01116 in 64 patients with breast cancer tissues was significantly higher than the expression of 30 normal breast tissues. **B**, The overall survival rate of breast cancer patients with high expression of LINC01116 was significantly lower than that of LL22NC03-N64E9. 1 low expression group. **C**, The expression of LINC01116 was positively correlated with TNM stage. **D**, LINC01116 expression was positively correlated with tumor size.

Table I. The correlation between the expression of LINC01116 in mammary glands and clinicopathological f

Clinicopathologic features	Number of cases	LINC01116 expression		p-va
		Low (n=32)	High (n=32)	
Age (years)				0.802
≤50	21	15	16	
>50	33	17	16	
Gender				0
Male	38	18	20	
Female	26	14	12	
Tumor size				8*
≤2CM	28	10	18	
>2CM	36	22	14	
TNM stage				0.0451*
I-II	30	19	1	
III-IV	34	13	21	
Lymph node metastasis				0.3087
Absent	26	11	15	
Present	38	21	17	

Screening Cell Lines and Interfering Sequences

Total RNA was extracted from breast c cell lines MCF-7, MDA-MB-21 and HCC38 s, as well as control cell line MCF-10A The rel expression of LINC01116 was detected by q PCR. The expression of LINC01116 was the h ghest in HCC38 cell line, so HCC3 ine was chosen for the subsequent interfer ment. The corresponding interference s uence w con- structed and transfected into H 38 cell Re- sults of transfection were show igur interference effect of si-LIN 01116 s the best.

Knockdown of LIN 116 Expression Can R e Viability of Breast Cance ells

CCK8 assay s wed tha 450 value of HCC38 cells tra fected with si-L 116 decre- ased compare ith those transfecte with si-NC negative co l, indi ating that knockdown of LINC0111 hibited e viability of HCC38 cells (Figure CK8 ults sho d that LINC01116 can increas bility of ast cancer cells.

Kn wn of 116 Expression Re ate the ning Ability of reast ncer Cells

Clon wed that the ability of cells clones was significantly suppressed com- pa that of the negative control after tran- sfec NC01116, which indicated that the inte e LINC01116 inhibited the cloning ility of HCC38 cells (Figure 3D).

6 Regulates its Target Gene, ESR1, as a Sponge of miR-145

After knockdown of LINC01116, miR-145 was found upregulated (Figure 4A). To identify INC01116 targets, websites of starBase[8] (http:// rbase.sysu.edu.cn/) and RegRNA (http://regr- a.mbc.nctu.edu.tw/html/prediction.html) were used to predict the loci where LINC01116 bind to miRNAs. The results indicated that miR-145 was found to have a binding site for LINC01116 in both websites (Figure 4B). To verify this pre- diction, luciferase activity assay was performed. After construction of pGL3-LINC01116-Wild and pGL3-LINC01116-Mut, miR-145 mimics or nc were co-transfected into breast cancer cell HCC38, and the results showed that miR-145 mimics reduced relative luciferase activity of pGL3- LINC01116-wild. However, there was no difference in relative luciferase activity between pGL3-LINC01116-Mut and the negative control (Figure 4C). Therefore, LINC01116 could bind directly to miR-145. Furthermore, the target ge- ne of miR-145 was predicted from the websi- tes (DIANA, miRanda, PicTar) and ESR1 was selected for further study (Figure 4D). It was demonstrated that miR-145 reduced the relative luciferase activity of ESR1 (Figure 4E, F), im-

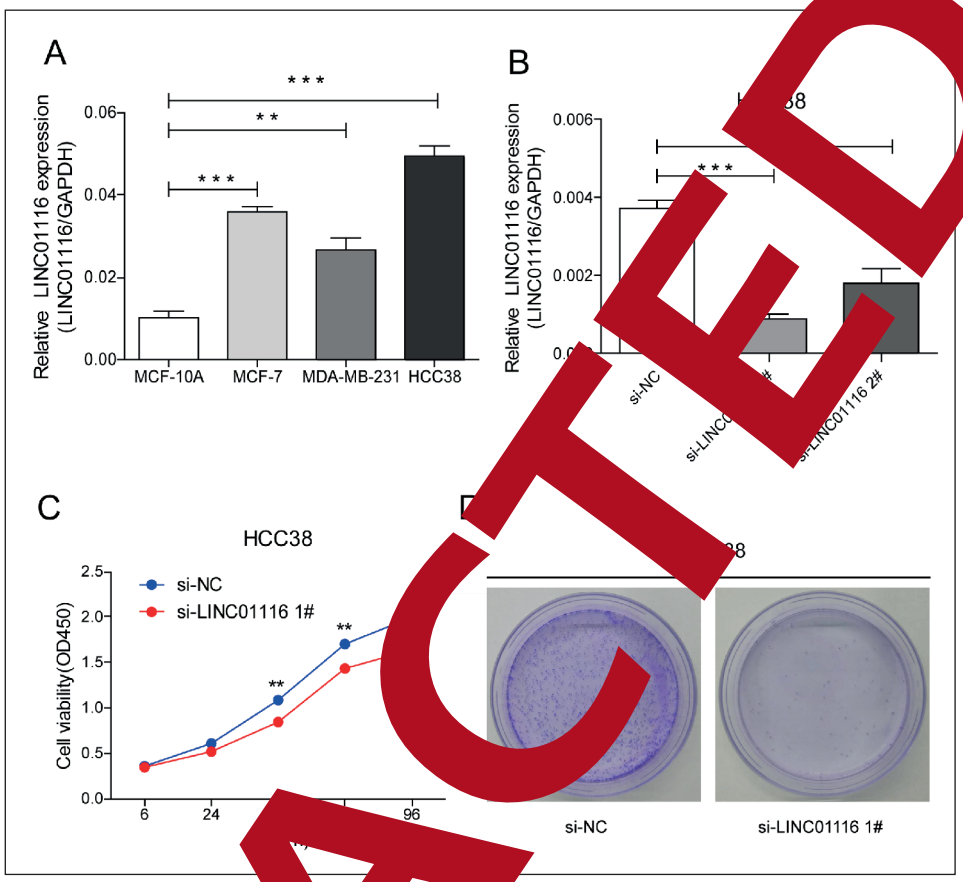

Figure 3. Effect of LINC01116 knockdown on cell phenotype. **A**, Expression of LINC01116 in breast normal and cancer cell lines (MCF-10A, MCF-7, MDA-MB-231, HCC38). **B**, The interference efficiency of si-RNA in HCC38 cells. **C**, CCK8 assay showed that interference with LINC01116 HCC38 cells inhibit the cell viability. **D**, Interference LINC01116 1 # reduced the proliferation of HCC38 cells.

plying that ESR1 was the target ge iR-145. Figure 4G and 4H showed that yn of LINC01116 or upregulation of -145 c redu-ce ESR1 expression. It was fu er demo trated that LINC01116 regulated its gene a sponge of miR-145.

Di on

The incidence f breast r ranks first among female' umors, among invasive ductal breast ost commonly obse ed with an increasing i dence very year. Although there are curre many tments for breast cancer, the cura ffect till uns factory. Therefore, to seek l cular ta s for breast cancer treatment is a ic i e current research.

e, we d ed the GSE54002 breast canc microarray ata set from the GEO database d analyzed the genetic map of breast cancer a reast tissue, and we found cRNA LIN 01116 was highly overexpressed ast cancer. Some studies have shown that LINC01116 had a proliferative promotion le in glioma[7] and prostate cancer[9]. However, w researches reported the relationship between INC01116 and breast cancer. We hypothesized that LINC01116 was a cancer-promoting oncogene. In this study, our clinical samples were used to detect the expression of LINC01116 in breast cancer. Also, interference of LINC01116 expression was performed for comparison. The results showed that, compared with normal breast tissue, the upregulation of LINC01116 in breast cancer was related to the size of large tumor and advanced stage of pathology. In addition, the overall survival of patients with high expression of LINC01116 was significantly shorter than that of patients with low expression of LINC01116. High expression of LINC01116 promoted the proliferation of breast cancer cells. It was suggested that LINC01116 can provide the basis for the prevention and target treatment of breast cancer. To date, many studies have supported the ceRNA hypothesis. For example, PTEN was an important tumor suppressor gene that was abnormally altered in a variety of human tumors.

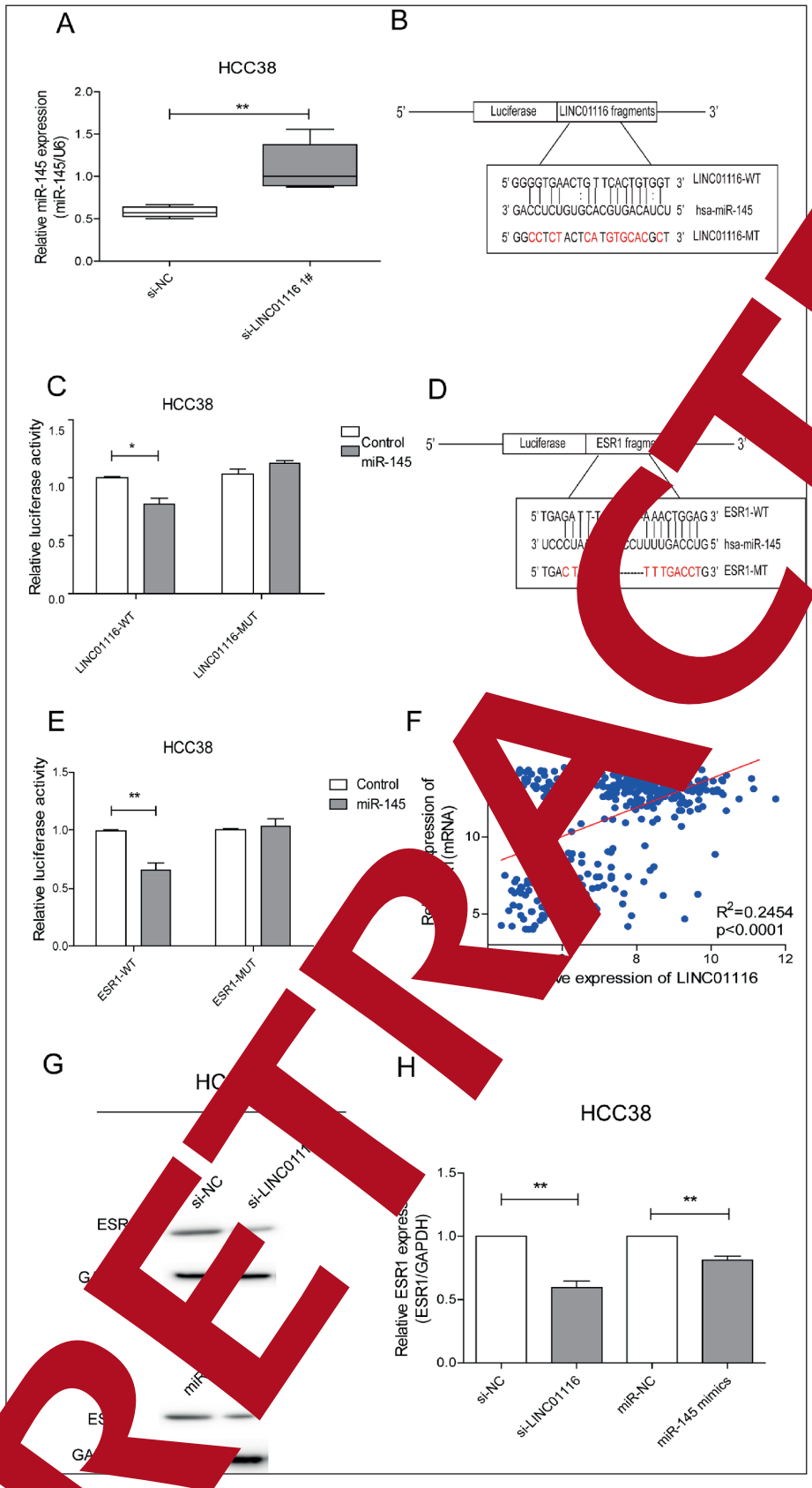

Figure 4. LI[illegible] lates ESR1 [illegible] miR-145. **A**, Expressio[illegible] f miR-145 in HCC38 [illegible]ter knockdown of LIN[illegible] **B**, Predicted binding sit[illegible] iR-145 and 3'-[illegible]TR regio[illegible] C0111[illegible] **C**, uciferase rep[illegible] wed whether [illegible] ound t[illegible] LINC01116. **D**, Predicti[illegible] binding sites for iR-1[illegible] and 3'-U[illegible] region [illegible]. **E**, L[illegible] rase re-p[illegible] ne s[illegible] d wheth-er i[illegible] d to ESR1. **F**, Co[illegible] analysis of LINC01116 [illegible]nd ESR1 expression levels. **G**, Protein expression of ESR1 after knock-[illegible] of LINC01116 and [illegible]pression of miR-145. **H**, ES[illegible] mRNA expression after knockdown of LINC01116 and overexpression of miR-145.

PTEN ceRNAs have been found in melanoma, prostate cancer and glioblastoma[10-12]. These findings confirmed the existence of intracellular regulation of ceRNA network, and their extensive involvement in tumor angiogenesis, invasion, metastasis and other processes[13,14]. Once the balance of the network of ceRNAs has been broken, it can lead to diseases, including tumors. In order to investigate the mechanism of LINC01116 in the pathogenesis of breast cancer, we selected miR-145 as a miRNA model to further study the target gene ESR1. ESR was involved in the occurrence and development of many tumors. Although ESR has two subtypes (ESR1 and ESR2), only ESR1 was highly expressed in normal pituitary and pituitary adenomas. Our study showed that in breast cancer cells, LINC01116 can act as a ceRNA and bind directly to miR-145, resulting in the up-regulation of ESR1. In summary, this study first demonstrated that LINC01116 was highly expressed in breast cancer, which can promote the proliferation of breast cancer cells Its high expression may be related to the progress of breast cancer, and was expected to become an independent prognostic indicator of breast cancer. In addition, LINC01116 can be used as an endogenous sponge to adsorb miR-145, leading to the up-regulation of miR-145 target gene ESR1, which led to the progression of breast cancer.

Conclusions

We showed that overexpression of LINC01116 is a biomarker of poor prognosis in breast cancer, providing a malignant phenotype for tumor cells. The ceRNA regulatory network of LINC01116 helps the better understanding of the pathogenesis of breast cancer. LINC01116 is expected to become a new prognostic marker and therapeutic target for breast cancer.

Conflict of interest

The authors declared no conflict of interest.

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