Linc00460/miR-641 promoted promotes proliferation and autophagy of breast cancer

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Research Article

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Abstract

This manuscript aimed to investigate the oncogenic role of linc00460 in breast cancer both in vivo and in vitro and then specify its downstream microRNAs to build a ceRNA network. As for in vivo tests, we used subgroup analysis and nomogram for survival analysis. As for in vitro tests, qRT-PCR, CCK-8 and Dual luciferase reporter gene were used. Linc00460 expressed highly in breast cancer. The nomogram indicated that linc00460 was associated with worse prognosis. Linc00460 might negatively regulate miR-641 to promote the proliferation and autophagy of breast cancer cell. In conclusion, linc00460 could be a risk factor for breast cancer by regulating miR-641; and it has the potential to be a novel biomarker.

Introduction

Breast cancer is the most common malignant cancer for women from all over the world[1]. Based on data from SEER (Surveillance, Epidemiology, and End Resluts), about 12.4% of women might be diagnosed with breast cancer during their lifetime [2]. The prognosis of breast cancer patients differed significantly according to different molecular subtypes based on the status of estrogen receptor ER, progesterone receptor PR, and HER2)[3,4]. Patients with presence of estrogen receptors, progesterone receptors or HER2 tended to have better prognosis based on the benefits of hormone therapy or targeting therapy of HER2. However, approximately 15%-25% of breast cancer did not express the ER, PR and HER2[3]. This molecular subtype was defined as triple-negative breast cancer (TNBC). This made treatment become more difficult because most therapies target one of the three receptors. And the recurrence and metastasis risks for TNBC patients were significantly higher than other breast cancer patients [5–7]. Therefore, it is important to find an efficient therapy targets for TNBC in order to improve their prognoses.

Long noncoding RNAs (lncRNA) have attracted great attention in recent years according to their various biological functions in cancers[8–10]. LncRNAs represent a group of RNA transcripts which are longer than 200 nucleotides but cannot be translated into proteins [11]. Previous study indicated that LncRNAs could function as competing endogenous RNA by binding to microRNA or proteins directly in cells. Increasing studies have demonstrated that LncRNAs are also involved in TNBC development[10]. For example, MIR100HG can promote the proliferation of TNBC cells by participating in the formation of RNA-DNA triplex structures[12]. LINC00460 has been found highly expressed in esophageal carcinoma, colorectal cancer, non-small lung cancer and so on[13–16]. These studies showed its roles in tumorigenesis. However, it's biological roles and molecular functions in TNBC remain elusive. In this study, we aimed to explore the molecular functions of LINC00460 in TNBC. Here, we found that LINC00460 could act as a novel biomarker for TNBC, of which patients with highly LINC00460 expression are prone to have poor prognoses. Besides, LINC00460 could promote TNBC cells proliferation abilities by sponging miR-641. Therefore, LINC00460 may provide a new insight for the TNBC treatments.

Methods

1. Breast cancer patients' tissues

Breast cancer tissues and corresponding adjacent normal tissues were obtained from 168 patients in First affiliated hospital of Xi'an Jiaotong University. All breast cancer patients were pathologically diagnosed. All tissues were reserved in liquid nitrogen immediately after resection. Informed consent was obtained from all patients who were involved in our study. Our study was approved by the ethnic committee of First affiliated hospital of Xi'an Jiaotong University. Our study was carried out in accordance with the declaration of Helsinki.

2 Cell culture and transfection

Breast cancer cell lines (MAD-MB-231 and MCF-7) were purchased from American Type Culture Collection (ATCC, Manassas, USA). Normal breast cell line, MCF-10a, was purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and incubated at 37°C and 5% CO_2.

The linc-00460 and miR-641 lentivirus and relative negative control lentivirus were synthesized by GenChem (Shanghai, China). Transfection was thereby performed according to the manufacturer's instructions.

3. Real-time PCR for detection of linc00460and miR-641

Total RNA was extracted by Trizol reagent (TAKARA, Japan). Breast cancer and para cancer tissues' total RNA was extracted by Trizol reagent (Carlsbad, USA).

As for linc00460, SYBR Premix Ex Taq II (Takara, Japan) was used for PCR reactions. GAPDH was used as the control.

As for miR-641, Taqman MirNA reverse transcription Kit (Carlsbad, USA) and PrimeScriptTM RT Master Mix (Takara, Japan) were used for reverse transcription. Then qPCR process was conducted by Taqman MirNA assay kit and SYBR® Select Master Mix. The relative expression of LINC00460 and miR-641 were normalized to GAPDH and U6 respectively, by using the $2^{-\Delta\Delta Ct}$ method.

U6

forward 5′-CTCGCTTCGGCAGCACA-3′,

reverse 5′- AACGCTTCACGAATTTGCGT-3′.

GAPDH

Forward 5′-TCGGAGTCAACGGATTTGGT-3′,

reverse 5′-TTGGAGGGATCTCGCTCCT-3′

LINC00460

forward 5′- GTGGATGAGAACGAAGGTTACG-3′,

reverse 5′- CTTTCCCACGCTCAGTCTTT – 3′

miR-641

forward 5′-UGGUGGGCCGCAGAACAUGUGC – 3′,

reverse 5′- ACAUGUUCUGCGGCCCACGAAU-3′

4. Dual luciferase reporter analysis

pmirGLO, pmirGLO-linc00460-WT and pmirGLO-linc00460-Mut(miR-641) were purchased from Genchem(Shanghai, China). Approximately 4000/well target cells were seeded in 96-well plates; cells were co-transfected with pmirGLO, pmirGLO-linc00460-WT and pmirGLO-linc00460-Mut. Then, Luciferase activity was measured 24 h after co-transfection.

5. Cell proliferation assay

Cell proliferation ability was evaluated by cell counting kit-8 (CCK-8) assay (Dojindo, Japan). Treated cells were seeded into 96-well plates with density of 1000 cells per well. 10 ul CCK8 reagent was added to each cell sample before absorbance measurement. The absorbance of each cell sample at 450 nm was measured at 0,1,2 days after CCK8 reagent added.

6. Protein extraction and western blot

RIPA buffer and BCA (Sigma Aldrich, Cambridge, MA) were used to extract and validate the total protein. Proteins were transferred onto the PVDF membrane after electrophoresis. After blocking the membrane for 2 hours, primary antibodies were incubated over night at 4℃. Secondary antibody was incubated for 1 hour at room temperature.

7. Autophagy

Autophagy virus (GFP-RFP-LC3) was synthesized by Genchem (Shanghai, China). Autophagy virus was transfected into target cells and cultured for at least one day; then, approximately 1×10^5 transfected cells were added into a 96-well plate. Confocal microscope was used to observe the green and red intensity.

8. Statistical analysis

The presenting Data was the mean ± SD from at least three independent experiments. Student's t-test was adopted to compare the difference between two groups. P value less than 0.05 was defined as statistically significant. All statistical analysis was calculated by R 3.3.5.

Results

1 linc00460 was up-regulated in breast cancer

As shown in Fig. 1A, the expression of linc00460 was higher in breast cancer tissues than that in para-cancer tissues. Based on Starbase V2.0, we have screened miR-641-3p, miR-503-5p, miR-320a, miR-320b, miR-491-5p and miR-3064-5p as linc00460's potential downstream target. We then found that miR-641 and miR-491 expressed lowly in breast cancer tissues (Fig. 1B).

2 linc00460 could negatively regulate the expression of miR-641

According to Table 1, we noticed that the expression of miR-641 and miR-491 statistic significantly correlated to the expression of linc00460. Furthermore, we down-regulated the expression of linc00460(Fig. 2A) in MDA-MB-231 cell line and found the correspondingly increased expression of miR-641 and miR-491 (Fig. 2B). Following dual luciferase reported gene assay indicated that only miR-641 had the potential to interact with linc00460(Fig. 2C and 2D).

Table 1
Clinical characteristics in low and high level linc00460 breast cancer patients

Clinical Manifestations	N	Low level linc-00460 (40)	High level linc-00460 (176)	P value
Stage				
I	91	24	67	0.052
II	97	14	83	
III	18	2	16	
IV	10	1	9	
ER				
(-)	28	6	22	0.671
(+)	188	34	154	
PR				
(-)	30	3	27	0.196
(+)	186	37	149	
Her-2				
(-)	185	34	151	0.897
(+)	31	6	25	
miR-641				
High	178	23	155	0.000*
Low	38	17	21	

* indicates that the P value was smaller than 0.05.

After that, we have up- and down- regulated miR-641 in MDA-MB-231 cell line. (Fig. 2E and 2F) The result showed that the exotic regulation of miR-641 wouldn't influence the expression of linc00460, suggesting that miR-641 could be the downstream target of linc00460 (Fig. 2G and 2H).

3 Survival analysis for linc00460 and miR-641 in breast cancer

As shown in Table 2, we found that high expression of linc-00460 was associated with worse overall survival (Fig. 3A); and high expression of miR-641 was associated with better overall survival (Fig. 3B). And advanced stage was associated with worse prognosis (Fig. 3C); while ER (Fig. 3D) and PR (Fig. 3E) were associated with better prognosis; However, the influence of Her-2 on overall survival was not statistically significant (Fig. 3F) (Table 2).

Table 2
Log-rank test for breast cancer patients.
* indicated that P < 0.05.

Variables	OS	P value
Stage		0.000*
I	50.17 ± 1.93	
II	42.41 ± 2.45	
III	29.46 ± 5.61	
IV	12.13 ± 2.78	
ER		0.003*
(-)	31.86 ± 4.62	
(+)	46.06 ± 1.59	
PR		0.002*
(-)	31.83 ± 3.61	
(+)	46.21 ± 1.62	
Her-2		0.082
(-)	45.39 ± 1.63	
(+)	36.94 ± 4.78	
miR-641		0.002*
High	41.99 ± 1.86	
Low	53.37 ± 2.05	
Linc00460		0.000*
High	55.31 ± 1.94	
Low	40.76 ± 1.88	

4 Nomogram for linc00460 and miR-641

In Table3, we found that advanced stage and high expression of linc-00460 were associated with worse prognosis. Therefore, we selected those independent risk factors to build a nomogram to predict the 1-year and 3-year survival rate for breast cancer patients. The nomogram indicated that M stage contribute the most to the prognosis of breast cancer (Fig. 4A); and linc00460 was an important risk factor as well. The calibration curve for 3-year overall survival prediction and 5-year (Fig. 4B) overall survival prediction

(Fig. 4C) and C-index (0.763 ± 0.061) suggested that the nomogram could precisely and correctly predict the survival rate.

Table 3
COX model for breast cancer patients.

Variables	HR	Inferior 95% CI	Upper 95% CI	P
Stage				0.047*
I	Reference			
II	2.542	1.399	4.618	
III	5.888	2.442	14.196	
IV	15.013	4.814	46.815	
ER				0.003*
(-)	Reference			
(+)	0.350	0.168	0.729	
PR				0.031*
(-)	Reference			
(+)	0.548	0.277	1.083	
Her-2				0.000*
(-)	Reference			
(+)	2.384	1.140	4.985	
miR-641				0.062
Low	Reference			
High	0.396	0.177	1.887	
linc00460				0.000
Low	Reference			
High	7.087	2.486	20.204	

5 linc00460 could promote proliferation and migration of breast cancer

The CCK-8 tests showed that down regulation of linc00460 would inhibit the proliferation of MDA-MB-231 cell line (Fig. 5A); and up regulation of miR-641 could inhibit the proliferation of MDA-MB-231 cell line,

while the down regulation of miR-641 promoted the proliferation (Fig. 5B and Fig. 5C). As for wound healing assay, we found that linc00460 downregulation leads to decreased wound closure (Fig. 5D). However, down regulation of miR-641 resulted in increased wound closure (Fig. 5E) whereas up regulation of miR-641 inhibit wound closure (Fig. 5F). Down regulation of miR-641 can reverse the inhibitory effect of linc00460 inhibition in breast cancer proliferation (Fig. 5G).

6 linc00460 and miR-641 promoted autophagy in breast cancer

We found that linc00460 knock down leads to decreased expression of LC3 II and increased expression of LC3 I, indicating that autophagy is suppressed (Fig. 6A). The average green luciferase intensity is significantly increased, indicating the autophagy is inhibited (Fig. 6B). Overexpression of miR-641 resulted in the suppression of autophagy as well (Fig. 6C). However, miR-641 knock down induces the expression of LC3 I and decrease the expression of LC3 II (Fig. 6D).

Discussion

The oncogenic role of linc00460 has been investigated in several cancers, including esophageal carcinoma, ovarian cancer, gastric cancer and lung cancer[17–19]; and we were the first to report its oncogenic function in breast cancer. Our group has found the aberrant high expression of linc00460 in breast cancer tissues, and further survival analysis showed that high expression of linc00460 led to the worse prognosis. Therefore, we assumed that linc00460 might have the potential to be a novel biomarker for breast cancer. Besides, we noticed the positively association of linc00460 expression with the presence of Her-2 status in vivo, which required more experiments to clarify the exact mechanisms. Based on current studies, linc00460 was involved in the progression of EMT process, which resulted in the invasive behavior of cancer cell[18]. In this context, it was more evident that linc00460 could regulate the malignant behavior of breast cancer. In addition, the most widely investigated molecular pathways for linc00460 was ceRNA. Xing H et al illustrated that linc00460 could sponge miR-539 and then induced the malignancy for meningioma[20]. Meanwhile, Ye J et al has reported that miR-302c-5p could be one of linc00460's targets using dual luciferase reporter gene[16].

As for survival analysis, we have built the nomogram including T stage, M stage, ER, PR, Her-2 and linc004600, to clearly and distinctly present the influence of every risk factor on breast cancer overall survival. For example, we could infer from the nomogram that linc00460 contributed more to the overall survival than miR-641 did. In addition, we could predict the 3- and 5- year survival rate exploiting the nomogram. For example, if there were a stage I, ER (-), PR (-), Her-2(-), linc00460(+) patient; according to the nomogram, the total score would be14.9, therefore the 3-year survival rate would roughly be 90%, and the 5-year survival rate would be around 35%. In short, linc00460 has the potential to be novel biomarkers in predicting the prognosis of breast cancer patients. And this conclusion needs a larger cohort for validation before it could be interpreted for clinical practices.

With the help of online informatics tool, Starbase V2.0, we were able to identify the potential downstream target, which was miR-641, for linc00460. To date, it is well known that miR-641 is located in the intronic region of AKT2, which is a vital oncogene for the apoptosis and chemotherapy response of carcinomas[21]. In glioma, miR-641 plays an important role in suppressing the activation of PI3K/AKT pathway, which is closely associated with tumor progression and development [21]. Moreover, Chen J et al has found that the overexpression of miR-641 was relevant of erlotinib resistance in lung cancer[22]. And miR-641 might regulate erlotinib resistance through negatively regulating NF1 and then inducing the expression of ERK pathway[22]. In the meantime, our further in vivo and in vitro experiments have presented the potential tumor suppressor role of miR-641 in breast cancer. In addition to our result, Rui Yao et al illustrated that miR-641 expressed lowly in lung cancer tissues and overexpression of it could result in the decreased proliferation and increased apoptosis rate [23]. And Kong Q et al found that miR-641 could function as a tumor suppressor through inhibiting MDM2 and inducing p53 expression in lung carcinoma as well[24]. However, the study focusing on the upstream regulator of miR-641 was scarce. In this manuscript, we have preliminary built a ceRNA network, linc00460/miR-641, which would shed light on the complex mechanisms for miR-641's oncogenic biofunctions and bring about novel therapeutic targets.

Declarations

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Compliance with the ethical standards

Conflicts of interest

The authors have declared that they have no conflicts of interest.

Ethics approval and consent to participate

The work was approved by the Ethics Committee of First affiliated hospital of xi`an jiaotong university . Each patient recruited was received and signed the Informed consent forms.

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Conflicts of interest

The authors have declared that they have no conflicts of interest.

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Figures

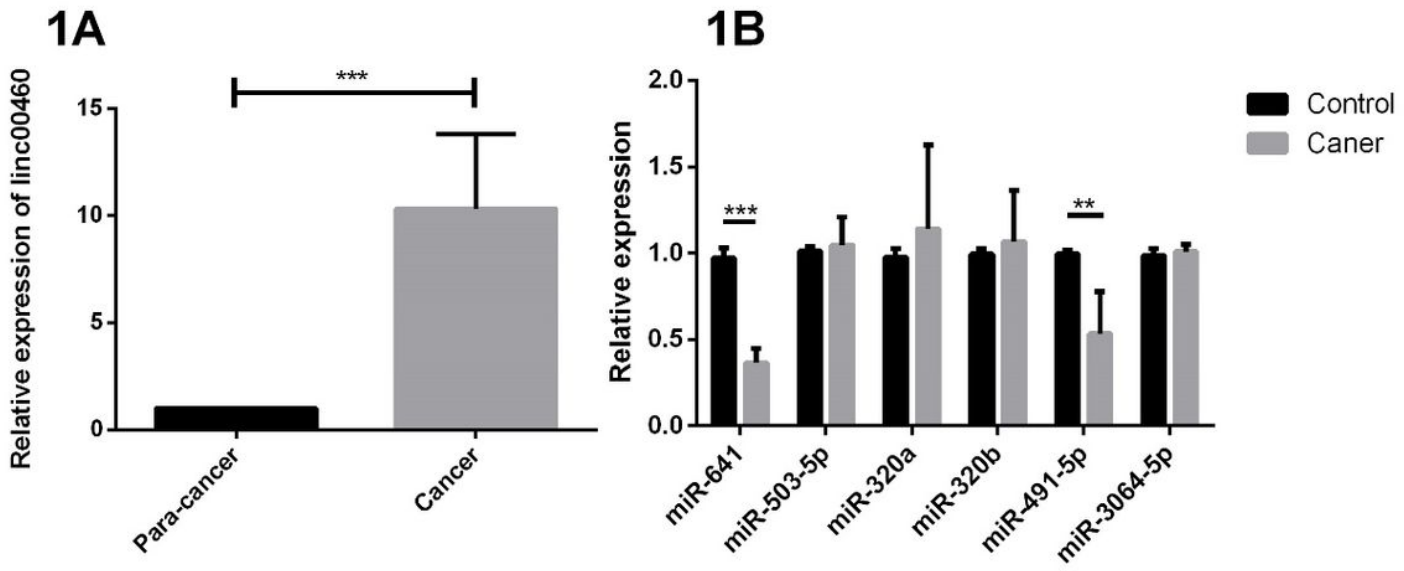

Figure 1

A The expression of linc00460 in breast cancer tissues. B The expression of microRNAs in breast cancer tissues; the control referred to para-cancer tissues.

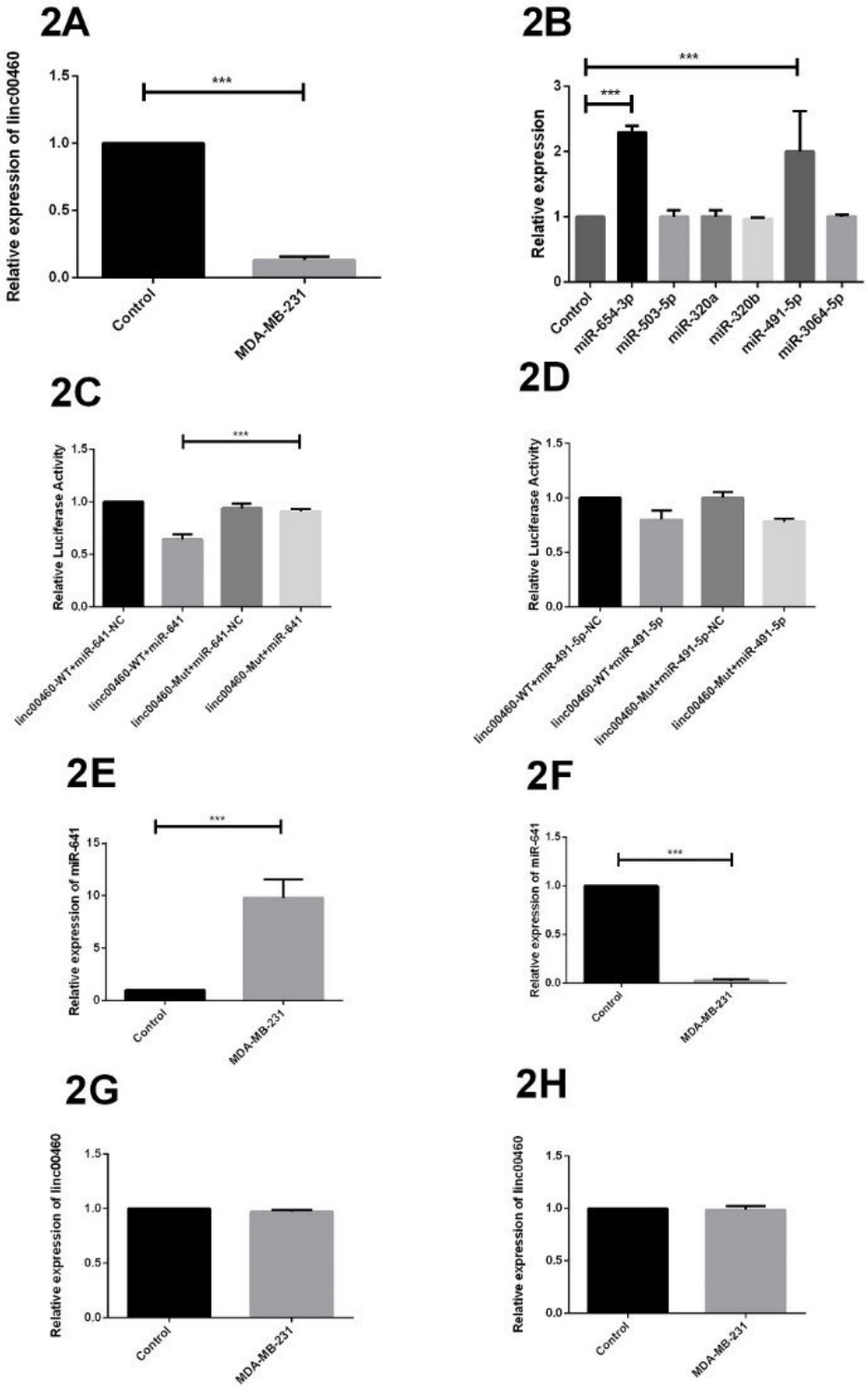

Figure 2

A The expression of linc00460 in MDA-MB-231 cell line. B The expression of microRNAs in MDA-MB-231 cell line. C Dual luciferase reporter gene for linc00460 and miR-641. D Dual luciferase reporter gene for linc00460 and miR-491.E The expression of up-regulation of miR-641 in MDA-MB-231 cell line. F The expression of down-regulation of miR-641 in MDA-MB-231 cell line. G,H The expression of linc00460 after up- and down- regulating of miR-641.

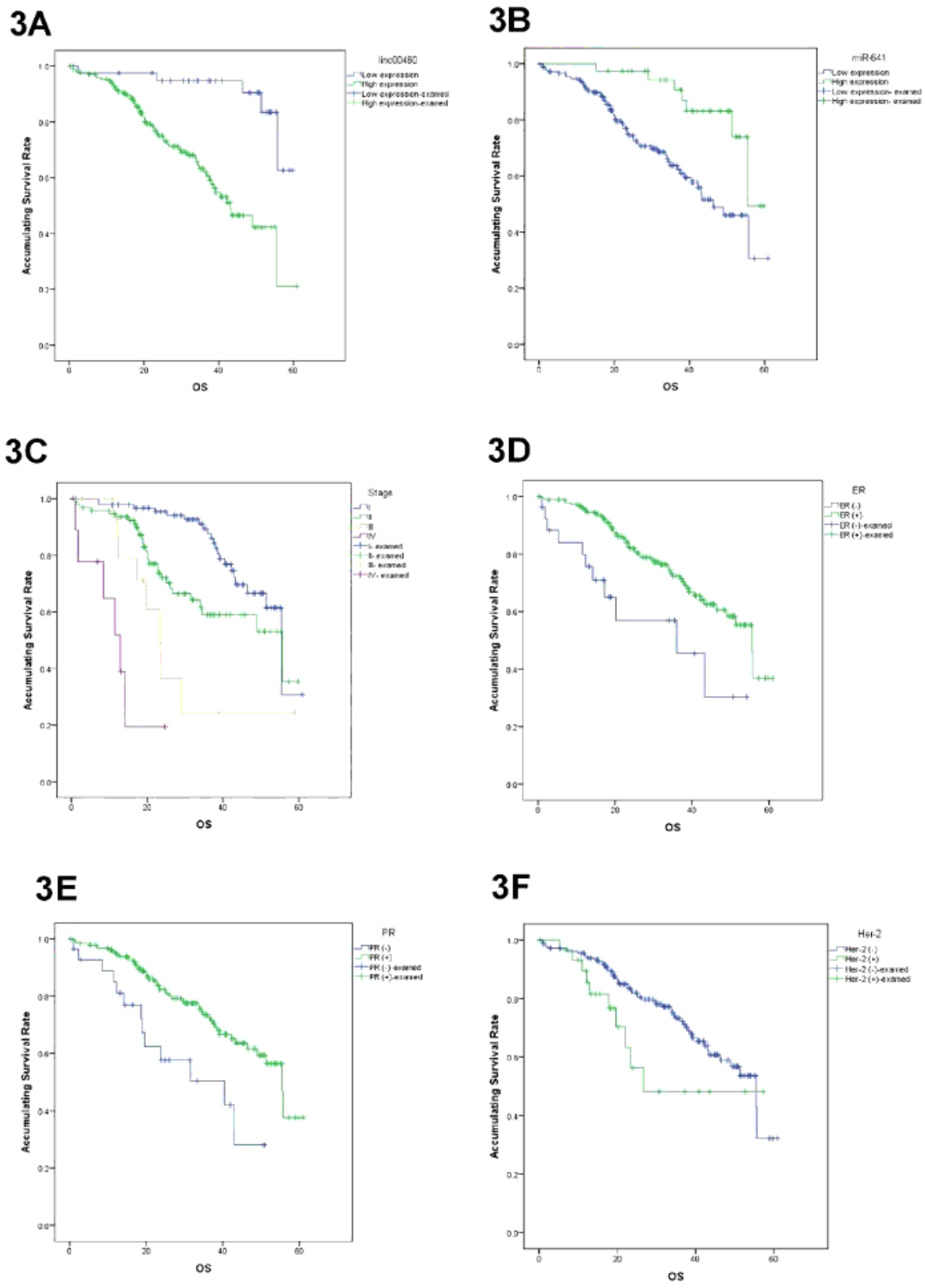

Figure 3

KM-plots drawn by SPSS 23.0. A Survival analysis for linc-00460 expression in breast cancer patients. B Survival analysis for miR-641 expression in breast cancer patients. C Survival analysis for different stages in breast cancer patients. D Survival analysis for ER status in breast cancer patients. E Survival analysis for PR status in breast cancer patients. F Survival analysis for Her-2 status in breast cancer patients.

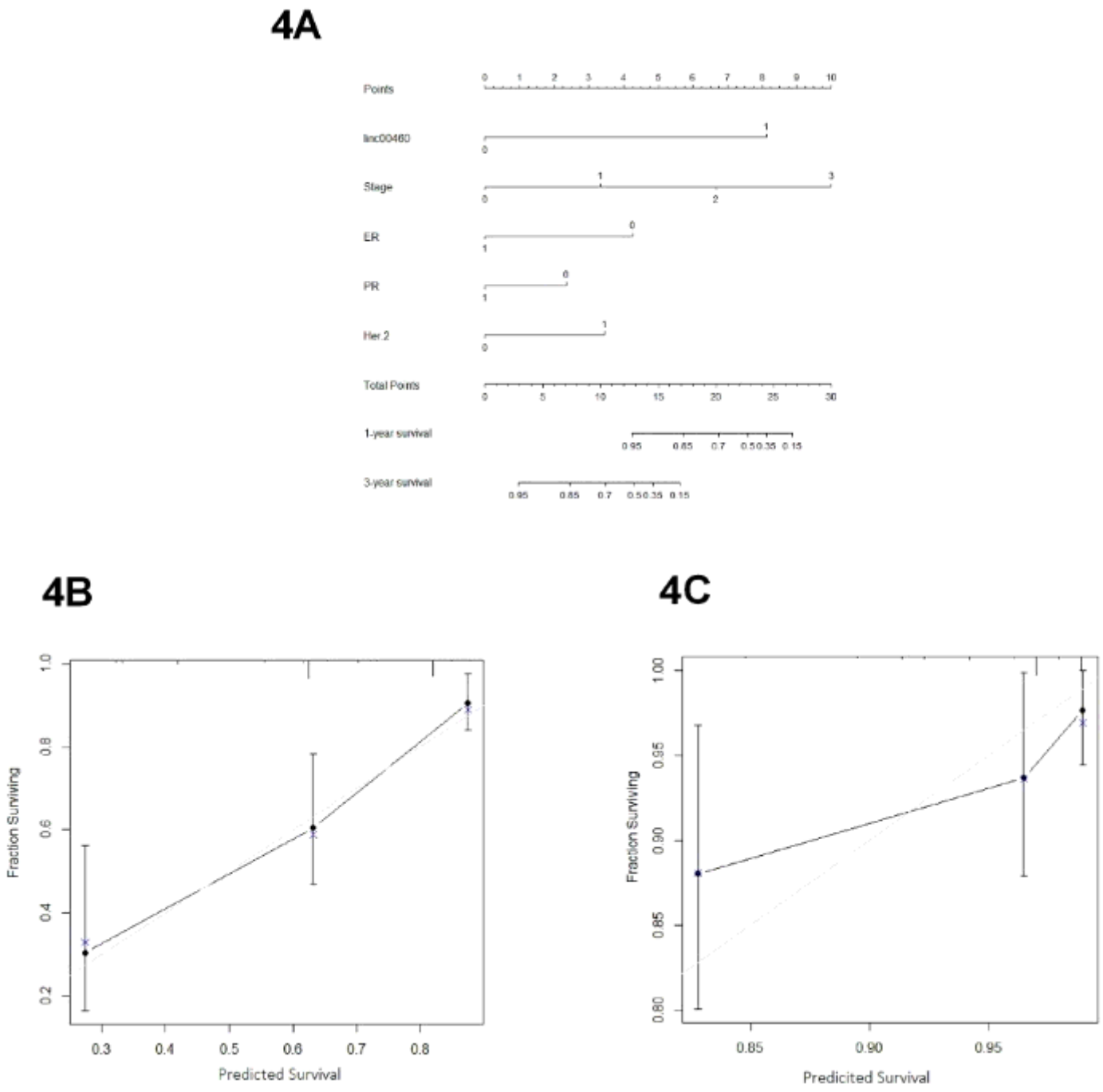

Figure 4

A Nomogram for breast cancer patients. B 1-year calibration curve. C 3-year calibration curve.

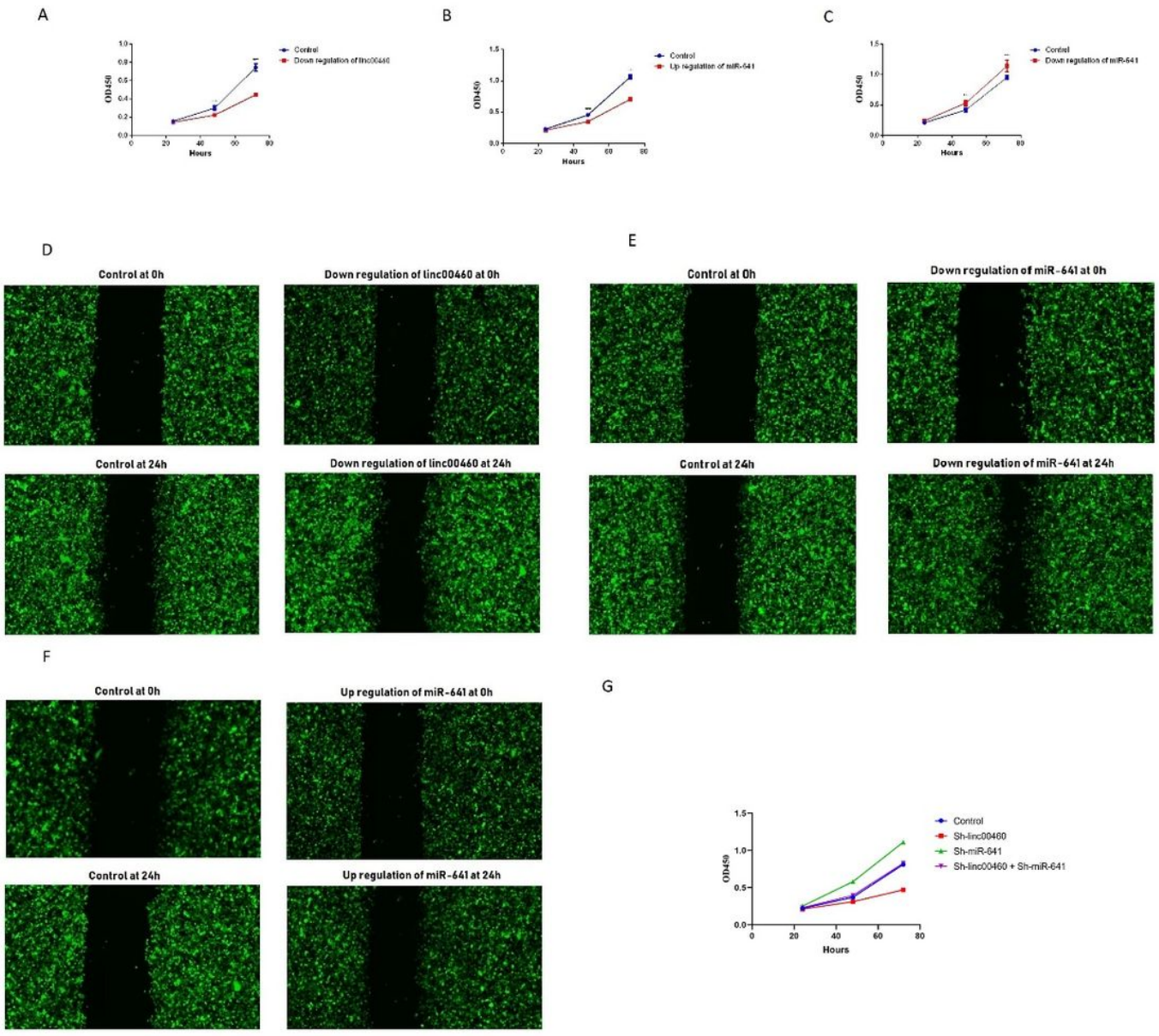

Figure 5

A CCK8 test after down regulation of linc-00460. B CCK8 test after up regulation of miR-641. C CCK8 test after down regulation of miR-641. D Wound healing assay for linc00460 down regulation. E Wound healing assay for miR-641 down regulation. F Wound healing assay for miR-641 up regulation. G CCK-8 results to show that miR-641 can reverse the inhibitory effect of linc00460.

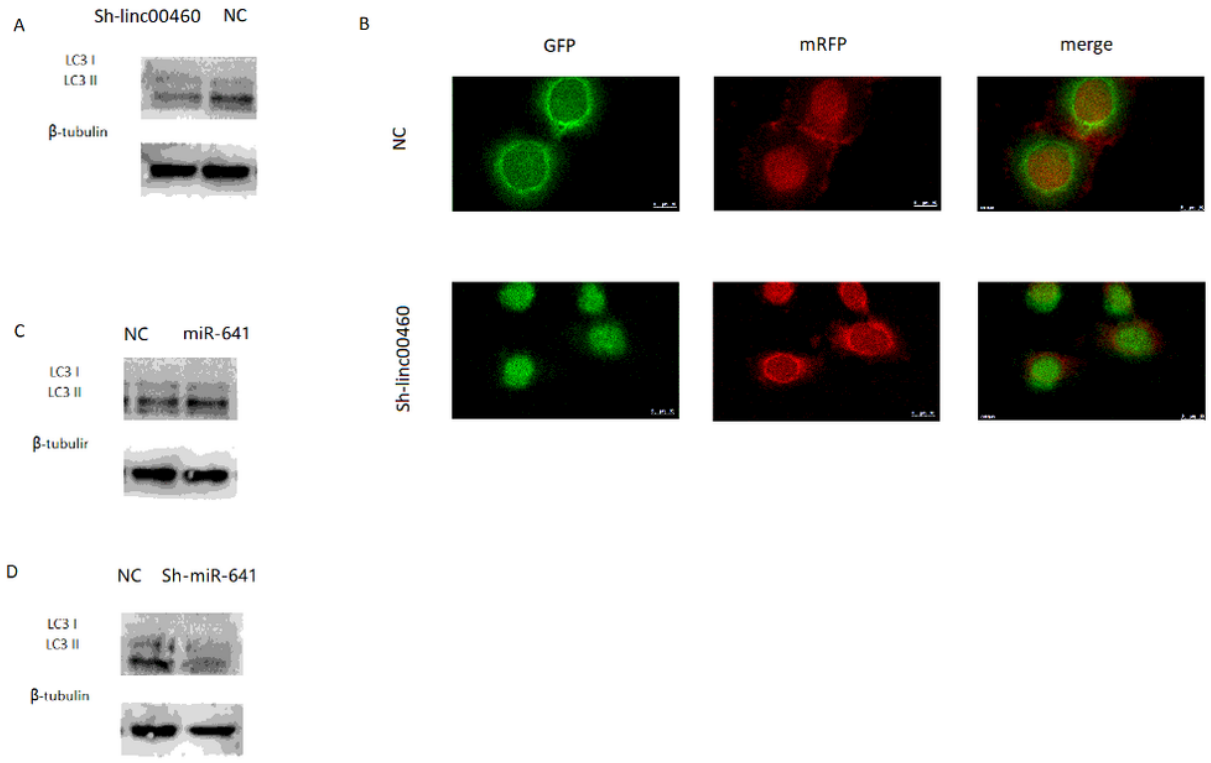

Figure 6

A LC3 I and LC3 II expression after downregulating linc00460 in MDA-MB-231. B The average green luciferase intensity is significantly increased, indicating the autophagy is inhibited. C LC3 I and LC3 II expression after upregulating miR-641 in MDA-MB-231. D. LC3 I and LC3 II expression after downregulating miR-641 in MDA-MB-231.