Diagnostic Value of LncRNAs for Postoperative Metastasis of Breast Cancer: A Nested Case-Control Study.

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Abstract

Background: Breast cancer is a malignancy with no clearly identified prognostic factors for diagnosis. Studies have preliminarily found that lncRNAs are related to breast cancer metastasis, however, the clinical predictive significance of lncRNAs is still elusive.In this study, we evaluated the diagnostic value of long non-coding RNA (lncRNA UCA1,CCAT2, ANCR) on postoperative metastasis of breast cancer as well as the possible mechanism involving the EMT.

Methods: We investigated lncRNA ANCR, UCA1,CCAT2 that associated with breast cancer metastasis risk in a population-based nested case-control study. Metastasis cases were identified by clinical diagnostic criteria in approximately 103 cases in the Cancer Institute of Southwest Medical University during 2013-2020. At the same time, the control group (metastasis-free) was selected according to the 1:1 pairing principle in this cohort (n=103, the matching condition was age±3 years, the operation time within the same month, and the treatment plan both are modifed radical mastectomy) The mRNA of lncRNA(UCA1,CCAT2, ANCR) expression was determined by Real-time PCR. The expression of E-cadherin, N-cadherin, and vimentin proteins was detected by Western blot. The migration and invasion of transfected cells were determined by the Transwell assay.

Results: lncRNA ANCR, UCA1, CCAT2 was significantly up-regulated in breast cancer cells and postoperative metastasis of breast cancer.CCAT2 (OR=1.024, 95% CI: 1.010, 1.039), UCA1(OR=1.025, 95% CI: 1.011, 1.039),ANCR(OR=1.055, 95% CI:1.001, 1.111)was the risk factor for postoperative metastasis of breast cancer. Furthermore , we used the ROC curve to detect the optimal critical values of CCAT2, UCA1, ANCR , the risk of metastasis in the CCAT2 high expression group was 2.297 times that of the low expression group (OR=2.297, 95% CI:1.427 ~ 3.695, P< 0.05). The risk of metastasis in the UCA1 high expression group was 2.032 times that of the low expression group (OR=2.032, 95% CI 1.282 ~ 3.218, P<0.05). We further observed that lncRNA UCA1, CCAT2, ANCR was down-regulated in MDA-231 cells by 48 h of siRNA transfection. LncRNAs UCA1, CCAT2, ANCR silencing significantly decreased the percentage of migration and invasion cells, down-regulated N-cadherin, and up-regulated E-cadherin and vimentin in MDA-231 cells.

Conclusions: Our data suggested that lncRNA CCAT2 , UCA1,ANCR was a novel molecule involved in postoperative metastasis of breast cancer, which has predictive value in patients with breast cancer metastasis.

Background

Breast cancer is one of the most common malignancy in women worldwide [1]. Despite comprehensive treatment including chemotherapy and surgical resection, the metastasis remains the underlying cause of death in poor prognosis of breast cancer patients [2]. Metastasis relies on an array of processes, such as the bilateral transition between epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET), promotion of cancer cell invasion, migration[3]. More and more clinical

investigations have demonstrated that several primary breast cancer markers have been identified to be related to breast cancer metastasis and prognostic, such as tumor size, Lymph node status, histological grade. As well as, the expression of estrogen receptor (ER) and progesterone receptor (PR) and the amplification of HER2/c-erbB2 are associated with breast cancer metastasis [4]. However, due to heterogeneous of this disease, the effective predictive ability is only in approximately 30%, An urgent need exists for identify prognostic biomarkers with high sensitivity and specificity that could improve prognostic predictions[5, 6].

Long noncoding RNAs (lncRNAs) are limited protein-coding transcripts with more than 200 nucleotides[7]. Emerging evidences have revealed that lncRNAs play an important role in the regulation of cell proliferation, differentiation, senescence, and carcinogenesis. The dysregulated expression of lncRNAs is associated with cancer metastasis and poor outcome. Y, O., et al fond that HOTAIR was significantly higher in cancerous tissues compared with normal mucosa, HOTAIR might be a predictive marker for patients with peritoneal metastasis[8]. Liwen Hu demonstrated that MALAT1 ware correlated with poor prognosis in ESCC patients by Kaplan-Meier analysis, which is involved in ESCC cancer metastasis and recurrence[9]. Nowadays, more and more studies indicate that LncRNAs are ideal diagnostic biomarkers and therapeutic targets. However, due to lack of epidemiological population research, especially metastasis case and no-metastasis case. lncRNAs have not been applied in clinical diagnostic tests.

In cancer, we found that UCA1, CCAT2, ANCR are closely related to cancer metastasis. Overexpresses of UCA1 might serve as a high potential biomarkers for predicting lymphnode metastasis and poor outcome in gastric cancer, thyroid cancer [10, 11]. Several studies further indicated that UCA1 affects EMT, Junhua Luo fond that UCA1 was significantly higher in bladder cancer tissues and downregulation of UCA1 might suppress the EMT in bladder cancer cells[12].For breast cancer, UCA1 modulated EMT procession in MDA-MB-231 cells, furthermore, upregulation of UCA1 increases invasiveness of breast cancer cells by regulating the Wnt/β-catenin signaling pathway[13].CCAT2 was firstly discovered in microsatellite-stable colorectal, which could have a key role in metastasis. However, the contributions of CCAT2 to breast cancer was still uncertain. In the current paper, Yi Cai revealed that abnormal expression of CCAT2 could promote breast tumor cell growth by regulating the Wnt signaling pathway. Compared tumor with non-tumor tissues, CCAT2 was overexpress, which may represent a valuable predictive marker of clinical outcomes[14]. ANCR is a novel LncRNA with minority research. Previous study has found that ANCR promotes EZH2 ubiquitination and degradation, which effect the invasion and metastasis of breast cancer cells[15], Li Z et al also suggested this physiological function breast cancer cell[16]. To further elucidate this functions of ANCR in breast cancer metastasis, Zhongwei Li et al find that ANCR participates in TGF-β1-induced EMT and suggested that ANCR is critical for breast cancer cells migration and tumor metastasis in vitro and vivo[17]. But no further study of diagnostic value of LncRNAs for postoperative metastasis of breast Cancer.

Due to ANCR, UCA1, CCAT2 are more studied in other cancer but few studies in breast cancer metastasis. So in this study, nested case–control study was used to explore the relation between lncRNA ANCR, UCA1,

CCAT2 and breast cancer metastasis, in order to provide theoretical support for clinical treatment and prognosis.

Materials And Methods

Sample

The patients were gather from the follow-up cohort of the Cancer Institute of Southwest Medical University. A cohort was collected from the Department of Breast Medicine, Affiliated Hospital of Southwest Medical University since January 2011.As of 2021, we have collected about 1,360 cases breast cancer patients. The metastatic cases and controls selected for this study were from this cohort. Patients with metastases during follow-up were included in the metastatic case group. Metastasis is defined as the movement of tumor cells away from the primary site to nearby or distal discontinuities and, in the process, spread into a visible, clinically relevant mass. At the same time, the control group was selected according to the 1:1 pairing principle (n = 103, age ± 3 years, operation time and treatment plan were consistent).The pathological data used in this study were from the Department of Pathology, Affiliated Hospital of Southwest Medical University. The data collected included pathological data, clinical data, treatment protocols, and paraffin specimens from breast cancer patients. After the preliminary diagnosis of breast cancer patients in the Affiliated Hospital of Southwest Medical University, the data were obtained from the Department of Pathology. The Parafn blocks used in this study were examined by pathologist Xiabin Li, and the samples were 100% tumor cells. [18]

Cell culture

Human breast cancer cell lines MDA-MB-231 were provided from the hospital of Southwest Medical University. Cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Transfection

The LncRNA(91794,91797,91800,91803) and control RNA(NC) were obtained from Gima Pharmaceutical technology co. LTD in Shanghai. MDA-MB-231Cells were cultured in six –well plates. The cells were transfected with LncRNA or control RNA after 48 hours, by using EndoFectin Max Transfection Reagent(Gima Pharmaceutical technology co. LTD ,Shanghai).All steps were according to the manufacturer's protocols. Cells were harvested after 48h for RT-PCR and Western blot analyses.All RNA oligoribonucleotides were obtained from Genepharma (Shanghai, P.R. China), and the sequences were shown in Table 1.

Transwell assay in vitro

Cell invasion assay was performed as described previously. Briefly, 2 × 104 of MDA-MB-231 cells were planted in each upper chamber of the transwell chamber containing 100 µl of serum-free DMEM medium. The lower chamber was filled with DMEM containing 20% FBS. After culturing for 24 hours, the non-

invading cells retained in the upper chambers were removed from the membranes with a cotton swab, and the migrated/invaded cells in the upper chambers, which attached to the reverse side of the membranes, were fixed, stained with 0.1% violet crystal dye and counted in five randomly selected fields (100×) under a phase contrast microscope. Each experiment was performed in triplicate.

Ethical issues: (1) Patients with informed consent to participate. (2) The study plan has been reviewed by the Biomedical Ethics Committee of Southwest Medical University, and it is considered to meet the ethical requirements of clinical research, and the study plan is approved. Application acceptance Number: XNYD2018001.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

MDA-MB-231 cells, The total RNA of MDA-MB-231 cells were extracted using the Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. cDNA was converted from total RNA by using a Reverse Transcription Kit (Takara, Dalian, China) according to the instructions.

FFPE sections,Depending on the size of the tissue sample, 1 or 2 paraffin sections (10 mm thick) were used for the isolation of RNA. The sections were cut and immediately placed in a 1.5 mL tube, in duplicates for each sample. The samples were then isolated using the RNeasy FFPE isolation kit #73504. RNA isolation was carried out in an RNase-free environment.

According to manufacturer's instructions. cDNA was reversely transcribed using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, Liaoning, China).Gene expression was performed with SYBR® Premix Ex Tap II (TaKaRa, Dalian, Liaoning, China) and data collection were carried out on a real-time thermal cycler qTOWER 2.0/2.2 (Analytik Jena, Germany) Relative gene expression was calculated using the $2 - \Delta\Delta CT$ method and the results were normalized with β-actin as an internal control. The sequences were shown in Table 2.

Western blot

Cells were lysed in RIPA buffer containing protease inhibitor (Beyotime, Shanghai, China). Protein samples were separated by SDS-PAGE,then transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with E-cadherin (Abcam, shangha, China), N-cadherin (Abcam, China), Vimentin (Abcam), and GADPH (Bioss, Beijing, China) primary antibodies. Protein expression was assessed by ECL chemiluminescent regents (millipore, USA) and quantified by densitometry (Image J software) and normalized to the corresponding GADPH bands.

Statistical analysis

All data were analyzed using SPSS 25.0 statistical software, and bilateral P values below 0.05 were considered statistically significant. Power test was $(1-\beta) = 0.9$ used by statistics. The continuous variables in this study were all non-normal distributions, using the Wilcoxon signed-rank test in univariate analysis, and using the median (Interquartile Range) description. The relation between LncRNA and breast cancer

metastasis was analyzed by McNemar's test, cox risk model and other statistical methods. The ROC curve was analyzed by MedCalc sofware.

Result

3.1 Effects on mRNA expression of lncRNAs

As showed in Figure 1, the mRNA expression of ANCR,UCA1,CCAT2 in breast cancer metastasis group were higher than those in control group(metastasis-free group) (P<0.05).

3.2 Expression of general pathological indicators in breast cancer patients

As shown in Table 3, HER2, E-Cad, Ki67, Molecular subtypes and lymph node metastasis in the metastasis group was higher than that the control group (metastasis-free)(P<0.10). Inversely, the ER of the metastasis group was lower than that of the control group (metastasis-free) (P<0.05).There was no significant difference in Age, PR, P53, Pathological type, Tumor size and WHO Grade between the two groups (P>0.10).

3.3 Cox Regression Analysis

In order to reduce the confounding bias, cox regression analysis was performed on variables related to prognosis in univariate analysis. The results of mRNA levels showed that the lymph node metastasis(*OR*=2.896, 95%CI:1.643~5.104, P<0.001), ANCR(*OR*=1.055, *95%CI:*1.001~1.111, P<0.05), UCA1(*OR*=1.025, *95%CI:*1.011~1.09, P<0.001), UCA1(*OR*=1.024, *95%CI:*1.010~1.038, P<0.001) were the risk factors for postoperative metastasis of breast cancer. The variable assignment table is shown in Table 4. For details, see Tables 5.

3.4 Diagnostic value of lncRNA ANCR, UCA1,CCAT2

We discovery that the mRNA expression of ANCR, UCA1, CCAT2 is correlated with the metastasis of breast cancer(P <0.05). However, the ΔΔCT is a continuous variable and there is no exact cut-off value for diagnosis. In order to further understand the role of ANCR, UCA1, CCAT2 in the prognosis of breast cancer metastasis. Hence , we used the ROC curve to study the optimal critical values of ANCR, UCA1, CCAT2, combined with the Youden index, we can conclude that ANCR (cut-off value = 6, Se = 76.70%, Sp = 79.61%), UCA1 (cut-off value = 6, Se = 78.64%, Sp = 79.61%), CCAT2 (cut-off value = 6, Se = 67.96%, Sp = 74.76%), suggesting the risk of metastasis will increases. As shown in Figure 2 and Table 7. On the ground of the cut-off value predicted by ROC curve, ANCR, UCA1, CCAT2 were divided into the high expression group and the low expression group according to the cut-off value, and the effects of UCA1, CCAT2 on breast cancer metastasis were verified again. Among them, the risk of metastasis in the UCA1 high expression group was 2.032 times that of the low expression group (OR=2.032 95%CI: 1.282~3.218, P <0.05). The risk of metastasis in the XRCC4 high expression group was 2.297times that of the low expression group (OR=2.297, 95%CI:1.427~ 3.695, P <0.05). As shown in Table 8.

3.4 transwell migration assays

To further examine the role of LncRNAs on metastasis, transwell assays were performed to compare the migration and invasion capabilities of MDA-MB-231 cells. The results revealed that Si-ANCR, Si-UCA1 Si-CCAT2 significantly reduced MDA-MB-231 cell migration and invasion compared with NC groups (Figure.3).

3.5 Western blot detection shows the EMT relative protein expression

Increasing evidence shows that the EMT is an important factor in migration and metastasis. Furthermore, we next verified whether EMT markers were altered in cell model. The expression of E-cadherin, N-cadherin and vimentin protein level was analyzed by Western blot. The results demonstrated that the expression of N-cadherin, and vimentin was decreased while E-cadherin expression was increased in si-CCAT2. In si-ANCR and si-UCA1 group, vimentin protein and E-cadherin was statistically significant (Figure.4). To varying degrees, lncRNA ANCR, UCA1, CCAT2 may act by regulating the epithelial–mesenchymal transition (EMT) pathway.

Discussion

Breast cancer is one of the most aggressive malignant disease in women worldwide. Although these therapeutic methods may prolong lifespan and alleviate patient suffering, the prognostic outcome for CCA remains unfavorable. With a high tendency to metastasize, approximately 30% of breast cancer patients will present metastases [19].Thus, it is urgent to find novel diagnostic and therapeutic targets. Accumulating evidence indicates that lncRNA is closely related to tumor metastasis. For example, Jinfeng Zheng et al found that multivariate analyses showed the high CCAT2 expression was a potential independent prognostic factor in prostate cancer patients [20]. Y Xu indicated that CCAT2 was upregulated in CCA tissues and cell lines, further multivariate Cox regression analyses confirmed that CCAT2 expression could be regarded as an independent factor for overall survival in CCA patients[21].For breast cancer, many authors suggest that CCAT2 was overexpress in tumor tissues or BC cells compared with adjacent normal tissues, but metastasis cases .[22,23].The result of UCA1were same in Li Y et al[23].it were much higher in the breast tumor tissues than in the peritumor normal tissues. Li, Yu and Mota, M [24,25]also found the similar results .So, identification of lncRNA as the prognosis biomarkers is particularly important for metastasis breast cancer. In this study, lncRNA CCAT2, UCA1, ANCR in breast cancer metastasis group were higher than those in control group (metastasis-free group. Cox regression analysis showed that the lymph node metastasis(*OR*=2.896, 95%CI:1.643~5.104, *P*<0.001),ANCR(*OR*=1.055,*95%CI:*1.001~1.111,*P*<0.05),UCA1(*OR*=1.025, *95%CI:*1.011~1.09, *P*<0.001), UCA1(*OR*=1.024,*95%CI:*1.010~1.038, *P*<0.001) were the risk factors for postoperative metastasis of breast cancer. In order to further understand the prognosis role of lncRNA CCAT2, UCA1, ANCR in metastasis of breast cancer, we also studied the best cut-of value of lncRNA CCAT2, UCA1, ANCR, The sensitivity of lncRNA CCAT2, UCA1, ANCR single detection is between 72.82~74.46%, the specifcity is between 66.20~87.38%, the Youden index is between 0.3883~0.6214, and in the cox regression of breast

cancer prognosis, the odds ratio of the lncRNA CCAT2, UCA1 is as high as 2.297 and 2.023. It can be seen that lncRNA CCAT2, UCA1, ANCR has clinical predicted value in metastasis of breast cancer.

Additionally, EMT is shown to be implicated in the invasion and migration in cancer We then determined the effect of CCAT2, ANCR, UCA1 in MDA-MB-231 cells. We found that downregulated expression of CCAT2, ANCR, UCA1 inhibited cell migration and invasion. EMT is a well-characterized process that facilitates invasion and metastatic dissemination of human cancers. Therefore, we examined potential target proteins associated with migration and invasion, such as EMT-related gene expression. we further investigated whether CCAT2, ANCR, UCA1 could modulate EMT of breast cancer cells. we found that, besides regulating migration, CCAT2, ANCR, UCA1 was involved in the pathogenesis of metastatic BC by regulating EMT, si-ANCR, si-CCAT2 increased E-cadherin and decreased N-cadherin and vimentin, These data suggest that CCAT2, UCA1 may modulate cell invasion by promoting EMT-related gene expression in breast cancer.

Conclusion

In summary, we firstly establishes that the CCAT2, ANCR, UCA1 expression is strikingly disorder underlying the metastasis of breast cancer. The postoperative metastasis of breast cancer could be effectively predicted when the CCAT2 (2−ΔΔCT score)>4.18, UCA1 (2−ΔΔCT score)>2.87 .It indicate that CCAT2, ANCR, UCA1 may play a key role as an indicator negative prognostic factor for patients with metastasis. We also exhibited that CCAT2, ANCR, UCA1 may be a potential inducement in EMT of breast cancer cells. However, the mechanism of lncRNA ANCR, UCA1,CCAT2 on the metastasis of breast cancer remains indistinct.These new findings suggest that CCAT2, ANCR ,UCA1 may be used as a potential prognostic and therapeutic target of the metastasis of breast cancer.

Declarations

Ethics approval and consent to participate

Ethical issues: (1) Patients with informed consent to participate. (2) The study plan has been reviewed by the Biomedical Ethics Committee of Southwest Medical University, and it is considered to meet the ethical requirements of clinical research, and the study plan is approved. Application acceptance Number: XNYD2018001.

Consent for publication

The authors consent for publication

Availability of data and material

The data and materials of this study are available from the corresponding authors for reasonable requests.

Competing interests

The authors declare no competing interests.

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Author contributions

YT designed and guided the study; LY.H performed western blot and PCR experiments and drafted the manuscript, Parafn blocks used in this study were examined by pathologist XBL. ZHT performed the transwell migration assays. YXY performed the statistical analysis; DYJ and BW and HSZ collected the samples. The authors read and approved the final manuscript.

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Tables

Table.1 The primers used for Transfection

Genes	Primer sequences
Negative control	sense 5’- UUCUCCGAACGUGUCACGUTT-3’
	Anti-sense 5’- ACGUGACACGUUCGGAGAATT-3’
CCAT2	sense 5’-CCUGCUCUUAUUGCAUGAUTT -3’
	Anti-sense 5’-AUCAUGCAAUAAGAGCAGGTT -3’
UCA1	sense 5’-GGCUUAGCAACAGGGAAUATT -3’
	Anti-sense 5’- UAUUCCCUGUUGCUAAGCCTT-3’
ANCR	sense 5’-CUGCAUUCCUGAACCGUUATT -3’
	Anti-sense 5’-UAACGGUUCAGGAAUGCAGTT -3’

Table.2 The primers used for quantitative real-time polymerase chain reaction

Genes	Primer sequences
β-actin	sense primer 5’-CCACGAAACTACCTTCAACTCC-3’
	Anti-sense 5’-GTGATCTCCTTCTGCATCCTGT -3’
CCAT2	sense primer 5’-TGCAATAAGAGCAGGAAAAGA-3’
	Anti-sense 5’-CCAAGAGGGAGGTATCAACAG -3’
UCA1	sense primer 5’-GCTTAGTGGCTGAAGACTGATGC -3’
	Anti-sense 5’- GTCCATTTGAGGCTGTAGAGTTTGA-3’
ANCR	sense primer 5’- GTGCAGTGCCACAGGACTAGA-3’
	Anti-sense 5’-TGTCCCTAACAGAATCCACCTCC -3’

Table. 3 Clinicopathologic feature of breast cancer patients [n(%)]

Variable	Total	Control(metastasis-free)	metastasis	*P*-value
	N=206	n=103	n=103	
Age				
<50	98	47(45.6)	51(49.5)	0.577†
≥50	108	56(54.4)	52(50.5)	
ER				
Negative	79	33(32.0)	46(44.7)	0.032*
Positive	127	70(68.0)	57(55.3)	
PR				
Negative	108	46(44.7)	62(60.2)	0.714*
Positive	98	57(55.3)	41(39.8)	
HER2				
-/+	138	68(66.0)	70(68.0)	0.001*
+++	68	35(34.0)	33(32.0)	
E-Cad				
Negative	24	14(13.6)	10(9.7)	0.001*
Positive	182	89(86.4)	93(90.3)	
P53				
Negative	107	58(56.3)	49(47.6)	0.757*
Positive	99	45(43.7)	54(52.4)	
Ki67				
<20	54	31(30.1)	23(22.3)	0.001*
≥20	152	72(69.9)	80(77.7)	
Molecular subtypes				
Luminal A	36	22(21.4)	14(13.6)	0.064†
Luminal B	55	27(26.2)	28(27.2)	
Luminal HER2	45	25(24.3)	20(19.4)	
HER2-enriched	23	10(9.7)	13(12.6)	

Basal-like	47	19(18.4)	28(27.2)	
Lymph node metastasis				
0	71	49(47.6)	22(21.4)	0.000†
1~3	43	27(26.2)	16(15.5)	
4~9	38	12(11.7)	26(25.2)	
≥10	54	15(14.6)	39(37.9)	
Pathological type				
Carcinoma in situ	11	7(6.8)	4(3.9)	0.405†
Non-specific invasive carcinoma	193	95(92.2)	98(95.1)	
Invasive special type carcinoma	2	1(1.0)	1(1.0)	
Tumor size				
<2cm	60	33(32.0)	27(26.2)	0.819†
≥2cm and ≤5cm	120	55(53.4)	65(63.1)	
>5cm	26	15(14.6)	11(10.7)	
WHO Grade				
	9	8(7.8)	1(1.0)	0.465†
	128	60(58.3)	68(66.0)	
	69	35(34.0)	34(33.0)	

*P values were calculated by pairwise comparisons from χ 2 test. †P values were calculated by comparisons of groups from rank sum test, since tumor size and WHO grade were not meet the normal distribution.

Table .4 The variable assignment of cox model.

Variable	Variable assignment
Outcome	0=control; 1=metastasis
ER	0=negative; 1=positive
HER	0=negative; 1=positive
E-Cad	0=negative; 1=positive
Ki67	1='<20'; 2='≥20'
Lymph node metastasis	0='0'; 1='1~3'; 2='4~9'; 3='≥10'

Table.5 Cox regression of lncRNA expression in metastasis of breast cancer

Variable	*B*	*S.E.*	*Wald*	*P-value*	*OR(95%CI)*
ER	-0.280	0.225	1.547	0.214	0.765(0.486,1.175)
HER	-0.343	0.229	2.250	0.134	0.710(0.453,1.111)
E-Cad	0.045	0.354	0.016	0.899	1.046(0.522,2.095)
Ki67	0.104	0.265	0.155	0.694	1.110(0.661,1.865)
Lymph node metastasis					
0	-	-	-	-	reference
1~3	0.234	0.339	0.478	0.490	1.264(0.651,2.454)
4~9	0.615	0.381	3.735	0.053	1.849(0.991,3.449)
≥10	1.063	0.289	13.524	<0.001	2.896(1.643,5.104)
ANCR	0.053	0.027	3.993	0.046	1.055(1.001,1.111)
UCA1	0.025	0.007	12.763	<0.001	1.025(1.011,1.09)
CCAT2	0.023	0.007	11.2	<0.001	1.024(1.010,1.038)

Table. 6 The variable assignment table of cox model afer ROC prediction grouping.

Variable	Variable assignment
Outcome	0=control; 1=metastasis
ER	0=negative; 1=positive
HER	0=negative; 1=positive
E-Cad	0=negative; 1=positive
Ki67	1='<20'; 2='≥20'
Lymph node metastasis	0='0'; 1='1~3'; 2='4~9'; 3='≥10'
ANCR	1='≤1.96'; 2='>1.96'
UCA1	1='≤2.87'; 2='>2.87'
CCAT2	1='≤4.18'; 2='>4.18'

Table.7 The best diagnostic value of ANCR, UCA1 and CCAT2

Indicator	Cut-off Value	Sensitivity(%)	Specificity(%)	Youden index	AUC	AUC (95% CI)	
ANCR	>1.96	72.82	66.02	0.3883	0.735	0.669	0.793
UCA1	>2.87	72.82	69.90	0.4272	0.788	0.726	0.842
CCAT2	>4.18	74.76	87.38	0.6214	0.902	0.853	0.939

Table. 8 Cox regression of lncRNA high expression and low expression in postoperative metastasis of breast cancer

Variable	B	S.E.	*Wald*	*P-value*	OR(95%CI)
ER	-0.054	0.216	0.061	0.804	0.948(0.621,1.447)
HER	-0.266	0.221	1.457	0.227	0.766(0.497,1.181)
E-Cad	-0.106	0.352	0.091	0.763	0.899(0.451,1.793)
Ki67	0.250	0.260	0.930	0.335	1.285(0.772,2.137)
Lymph node metastasis	0.987	0.284	11.839	<0.001	2.660(1.523,4.643)
ANCR	0.377	0.247	2.332	0.127	1.458(0.899,2.365)
UCA1	0.709	0.235	9.118	0.003	2.032(1.282,3.218)
CCAT2	0.831	0.243	11.740	<0.001	2.297(1.427,3.695)

Figures

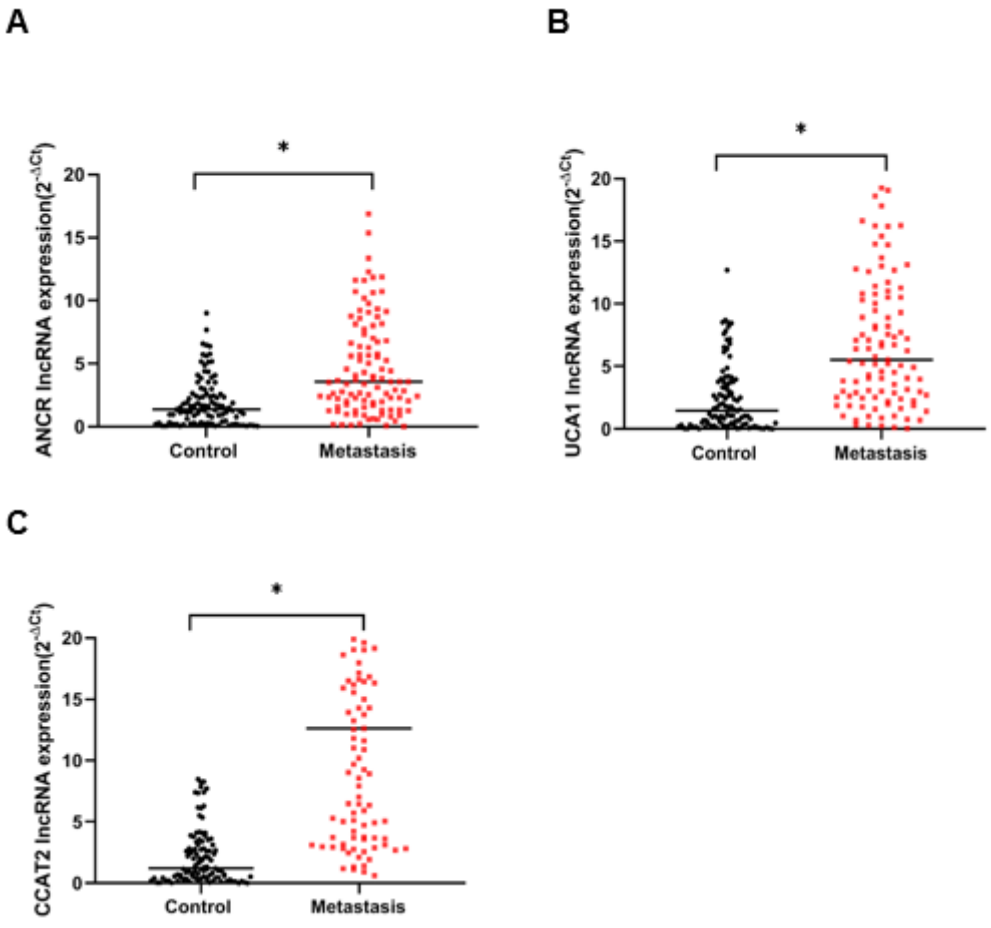

Figure 1

Shows the effect of breast cancer metastasis on the mRNA expression of lncRNA ANCR (A) , UCA1(B), CCAT2(C) . Data are described as Median (IQR), N=206. Statistical differences are expressed as: *P<0.05.

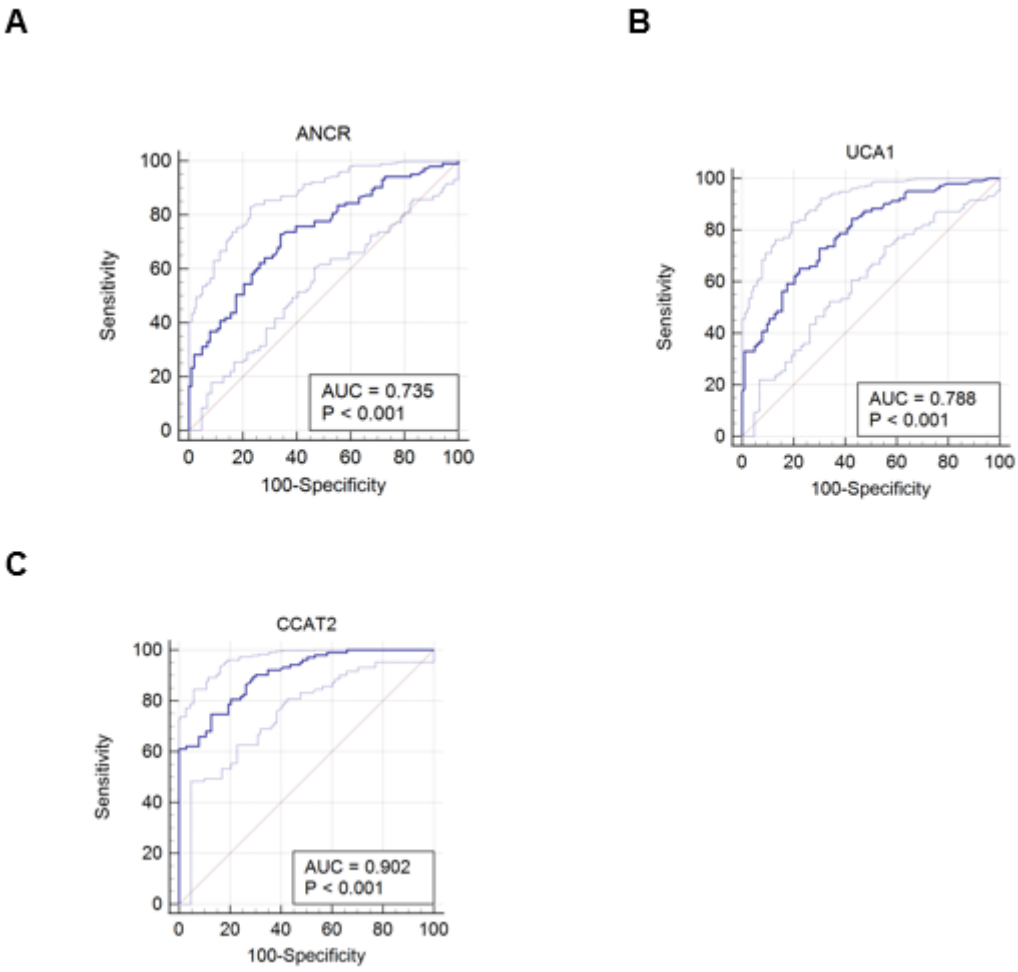

Figure 2

Diagnostic ROC curves of LncRNA expression. Diagnostic ROC curves of ANCR (A) ; UCA1(B);CCAT2(C);

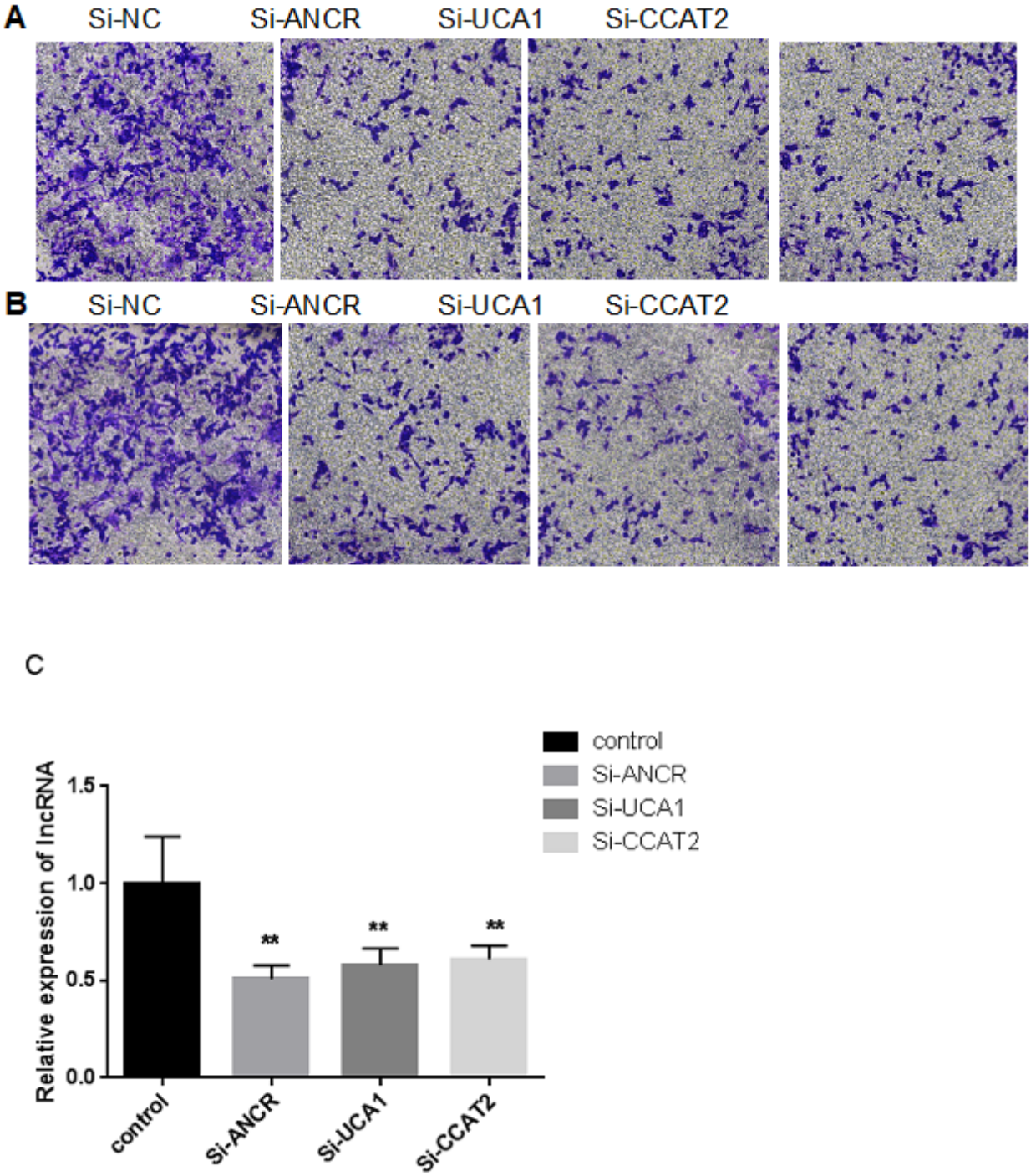

Figure 3

Transwell analyses showed the effects of LncRNA ANCR, CCAT2, UCA1 on MDA-MB-231 cells (magnification, 100X). A, inhibition of migration of MDA-MB-231 cells by siRNA. B, inhibition of invasion of MDA-MB-231 cells by siRNA. C, the relative expression level of LncRNA in MDA-MB-231cells Data are means ± SD. **, P < 0.05.versus control.

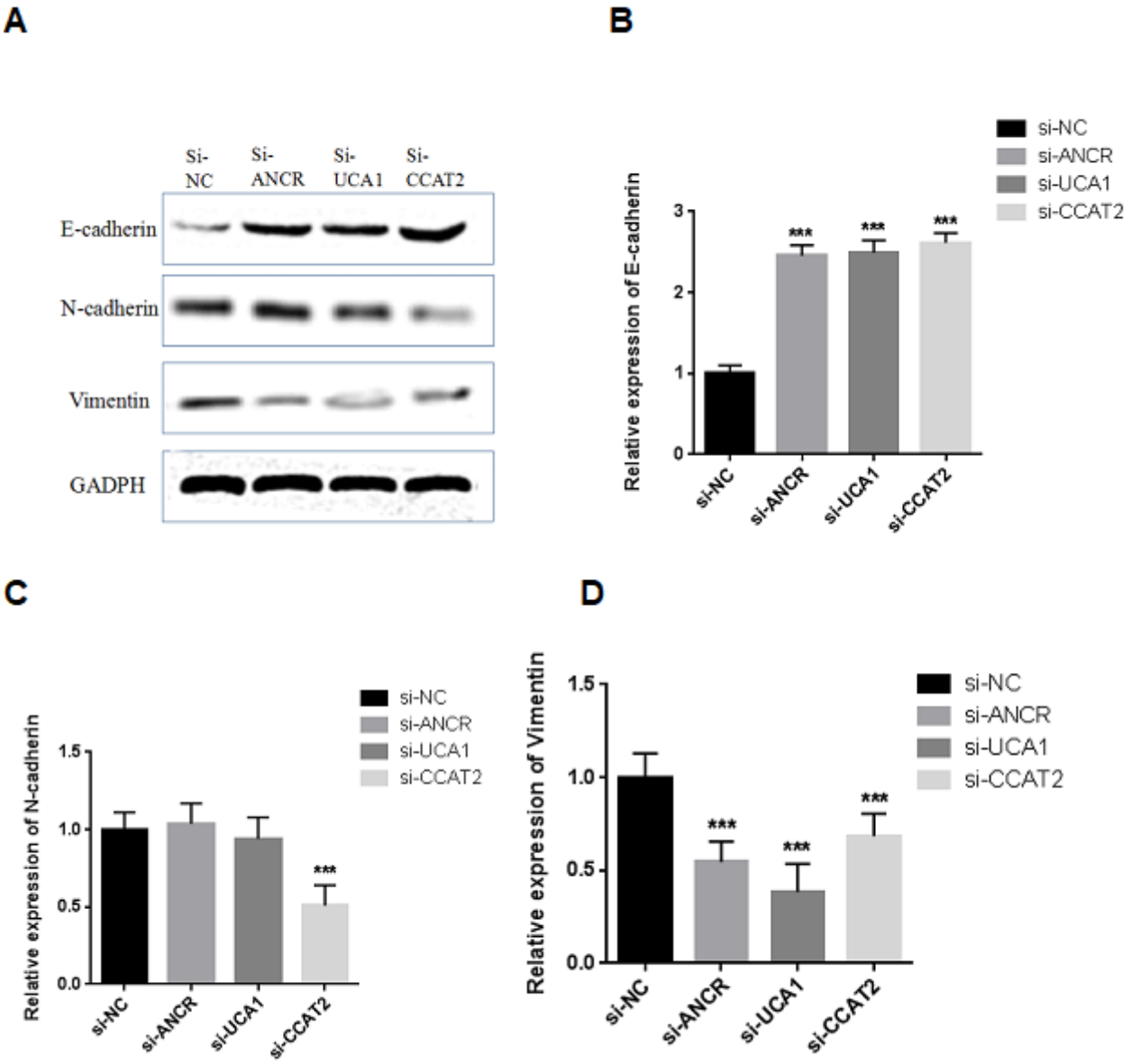

Figure 4

Western blot detection shows the relative protein expression. A, Western blot detection shows the relative protein expression. B, E-cadherin protein expression. B, N-cadherin protein expression. C, Vimentin protein expression. Data are means ± SD. **, P < 0.05.versus control.