

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

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Abstract

Background: Breast cancer is an aggressive tumor with no definitely identified prognostic biomarker for diagnosis. Studies have preliminarily found that lncRNAs are closely related to breast cancer metastasis, but, the significant clinical prediction of lncRNAs was remain unclear. 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 are at high stake of breast cancer metastasis 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. Meanwhile, the control group (no-metastasis) was single out on the basis of the 1:1 pairing principle in this cohort (n=103, the matching condition was the surgery time within the same month, and the treatment plan both are modified radical mastectomy ,age \pm 3 years) The mRNA of lncRNA(UCA1,CCAT2, ANCR) expression was determined by Real-time PCR. By Western blot, the expression of E-cadherin, N-cadherin, and vimentin proteins was detected. 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. Further more , By the ROC curve assay, we 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 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 the most aggressive tumor in women worldwide[1]. Despite comprehensive treatment including chemotherapy and surgical resection, Evidence has proved that the breast cancer patients with metastasis were at high risk of death in poor prognosis[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 prognostic biomarkers are 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 connected 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 longer than 200 nucleotides[7]. Emerging evidences have revealed that lncRNAs play a key role in the regulation of cell proliferation, differentiation, senescence, and carcinogenesis. The dysregulated expression of lncRNAs is connected with cancer metastasis and poor outcome. Y, O., et al found 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 were 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 may act as ideal diagnostic biomarkers. 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 et.al[10, 11]. Several studies further indicated that UCA1 affects EMT, Junhua Luo found 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 participating in 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 mechanism of CCAT2 to breast cancer metastasis was still unclear. In the current paper, Yi Cai revealed that abnormal CCAT2 could accelerate breast tumor cell growth by 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- β 1-induced EMT, indicated ANCR may be 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 cell biology but few studies in population-based metastasis. So, we conduct the nested case-control study to explore the relation between lncRNA ANCR, UCA1, CCAT2 and breast cancer metastasis, in order to provide the theoretical support for clinical medicine treatment and prognosis.

Materials And Methods

Sample

The metastatic cases and controls selected for this study were from Cancer Institute of Southwest Medical University. In our preliminary work, Yongxin Yang expounded specific collection methods[18]. Metastasis cases were identified by clinical diagnostic criteria in 103 cases. Meanwhile, the control group (no-metastasis) was select out on the basis of the 1:1 pairing principle in this cohort (n = 103, the matching condition was the surgery time within the same month, and the treatment plan both are modified radical mastectomy ,age \pm 3 years). All the samples were acquired with the patients' informed consent. Application acceptance Number: XNYD2018001.

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 and cultured at 37°C in a humidified atmosphere with 5% CO₂.

Transfection

The LncRNA(91794,91797,91800,91803) and control RNA(NC) were obtained from

Gima Pharmaceutical technology co. LTD in Shanghai. MDA-MB-231 Cells 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 in line with 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 se-quences were shown in Table 1.

Transwell assay in vitro

The invasion assay was performed as described previously[4]. Briefly, of MDA-MB-231 cells(2×10^4)were suspended in each upper chamber of the transwell chamber containing serum-free DMEM medium. The lower chamber was full of DMEM containing 20% FBS. For 24 hours at 37°C, the non-invading cells were stuck in the upper chambers. 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 \times) under a phase contrast microscope. Each experiment was conduct in triplicate.

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

MDA-MB-231 cells: The total RNA were performed by using the Trizol reagent (Invitrogen, Shanghai, China). cDNA was synthesised by using a Reverse Transcription Kit (Takara, Dalian, China)

- FFPE sections: Depending on the size of the tumor tissues, 1 or 2 paraffin sections (10 mm thick) were used for the isolation of total RNA. The sections were cut by Li Xiabin, the sample was full of tumor cells. The samples were then isolated using the RNeasy FFPE isolation kit #73504.

According to manufacturer's instructions. We conduct the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, Liaoning, China) and SYBR® Premix Ex Tap II (TaKaRa, Dalian, Liaoning, China) for RT-PCR. The data collection were performed by qTOWER 2.0/2.2 (Analytik Jena, Germany). the $2^{-\Delta\Delta CT}$ method was used to analyse the data and the results were normalized with β -actin. The primers used 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, Shanghai, China), N-cadherin (Abcam, China), Vimentin (Abcam), and GADPH (Bioss, Beijing, China) primary antibodies. Protein expression was assessed by ECL chemiluminescent reagents (Millipore, USA) and quantified by densitometry (Image J software) and normalized to the corresponding GADPH bands.

Statistical analysis

Data were processed with SPSS 25.0, and bilateral $P < 0.05$ were considered statistically significant. Power test was $(1-\beta) = 0.9$ used by statistics. In this study, the continuous variables were all non-normal distributions, Univariate analysis used the Wilcoxon signed-rank test and the median (Interquartile Range) description. The connection between lncRNA and breast cancer metastasis was analyzed by McNemar's test, cox risk model. The ROC curve was conducted by MedCalc software.

Result

3.1 Effects on mRNA expression of lncRNAs

Metastasis are crucial reasons for the poor prognosis of breast cancer. lncRNA ANCR, UCA1, CCAT2 play a crucial role in tumor progression. In our study, the mRNA expression of ANCR, UCA1, CCAT2 in breast cancer metastasis group were statistically exceeded those in control group (no-metastasis group) ($P < 0.05$), see Fig. 1.

3.2 Cox Regression Analysis

In previous study, ER, HER2, E-Cad, Ki67 and lymph node were considered statistically significant [18]. In order to reduce the confounding bias, cox regression analysis was performed on variables related to prognosis in univariate analysis. The data showed that the lymph node metastasis ($OR = 2.896$, $95\%CI: 1.643 \sim 5.104$, $P < 0.001$), ANCR ($OR = 1.055$, $95\%CI: 1.001 \sim 1.111$, $P < 0.05$), UCA1 ($OR = 1.025$, $95\%CI: 1.011 \sim 1.09$, $P < 0.001$), UCA1 ($OR = 1.024$, $95\%CI: 1.010 \sim 1.038$, $P < 0.001$) were at risk for postoperative metastasis of breast cancer., see Table 3.

3.3 Diagnostic value of lncRNA ANCR, UCA1, CCAT2

We discovered that the mRNA expression of ANCR, UCA1, CCAT2 is correlated with the metastasis of breast cancer. However, the $\Delta\Delta CT$ is a continuous variable, there is no exact cut-off value for breast cancer diagnosis. To further explicit the role of ANCR, UCA1, CCAT2 in the prognosis of breast cancer metastasis. Hence, we conducted the ROC curve to explore the optimal critical values of ANCR, UCA1, CCAT2, table 4 shows the variable assignment table of cox model after ROC prediction grouping. 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 increases. The data were shown in Fig. 2 and Table 5. 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 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). See in Table 6.

3.4 transwell migration assays

To further examine the role of lncRNAs on metastasis, transwell assays were performed to assay 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 (Fig. 3).

3.5 Western blot detection shows the EMT relative protein expression

Increasing evidence shows that the EMT play a key role in migration and metastasis. Furthermore, we verified whether EMT key factors were altered in cell model. The expression of key makers E-cadherin, N-cadherin and vimentin protein level was analyzed by Western blot, as can be seen in Fig. 4. 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. To varying degrees, lncRNA ANCR, UCA1, CCAT2 may participate in the EMT pathway.

Discussion

Breast cancer is one of the most aggressive malignant disease in women. Although these therapeutic methods may make for extending lifespan and alleviating patient suffering, the prognostic outcome for CCA remains unsatisfactory. 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 useful

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 overexpressed in tumor tissues or BC cells compared with adjacent normal tissues, but metastasis cases .[22, 23].The result of UCA1 were 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($OR = 2.896$, $95\%CI:1.643 \sim 5.104$, $P < 0.001$),ANCR($OR = 1.055$, $95\%CI:1.001 \sim 1.111$, $P < 0.05$),UCA1($OR = 1.025$, $95\%CI:1.011 \sim 1.09$, $P < 0.001$), UCA1($OR = 1.024$, $95\%CI:1.010 \sim 1.038$, $P < 0.001$) 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 specificity 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 dysregulated underlying the metastasis of breast cancer. The postoperative metastasis of breast cancer could be at high metastasis risk when the CCAT2 ($2 - \Delta\Delta CT$ score) > 4.18 , UCA1 ($2 - \Delta\Delta CT$ 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

Ethical Approval and Consent to participate

This work obtained ethics approval to use the human tissue samples by the the first affiliated hospital of southwest medical university. Informed consent was obtained from all individuals prior to surgery to use their tissue materials in research. All patients provided written informed consent. Application acceptance Number: XNYD2018001

Consent for publication

Not applicable

Availability of supporting data

Data generated during this study are included in this published article and datasets generated and analyzed supporting the findings of this study are available from the corresponding authors upon reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Y.-T. designed, guided the study; L.Y.-H, X.B.-L. and Z.H-T performed the experiments; Y.X.-Y. analyzed the data and wrote the paper; D.Y.-J. and B.-W and H.S.-Z 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'-CUGCAUUCUGAACCGUUATT -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 Cox regression of lncRNA expression in metastasis of breast cancer

Variable	<i>B</i>	<i>S.E.</i>	<i>Wald</i>	<i>P-value</i>	<i>OR(95%CI)</i>
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 4 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 5 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 6 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

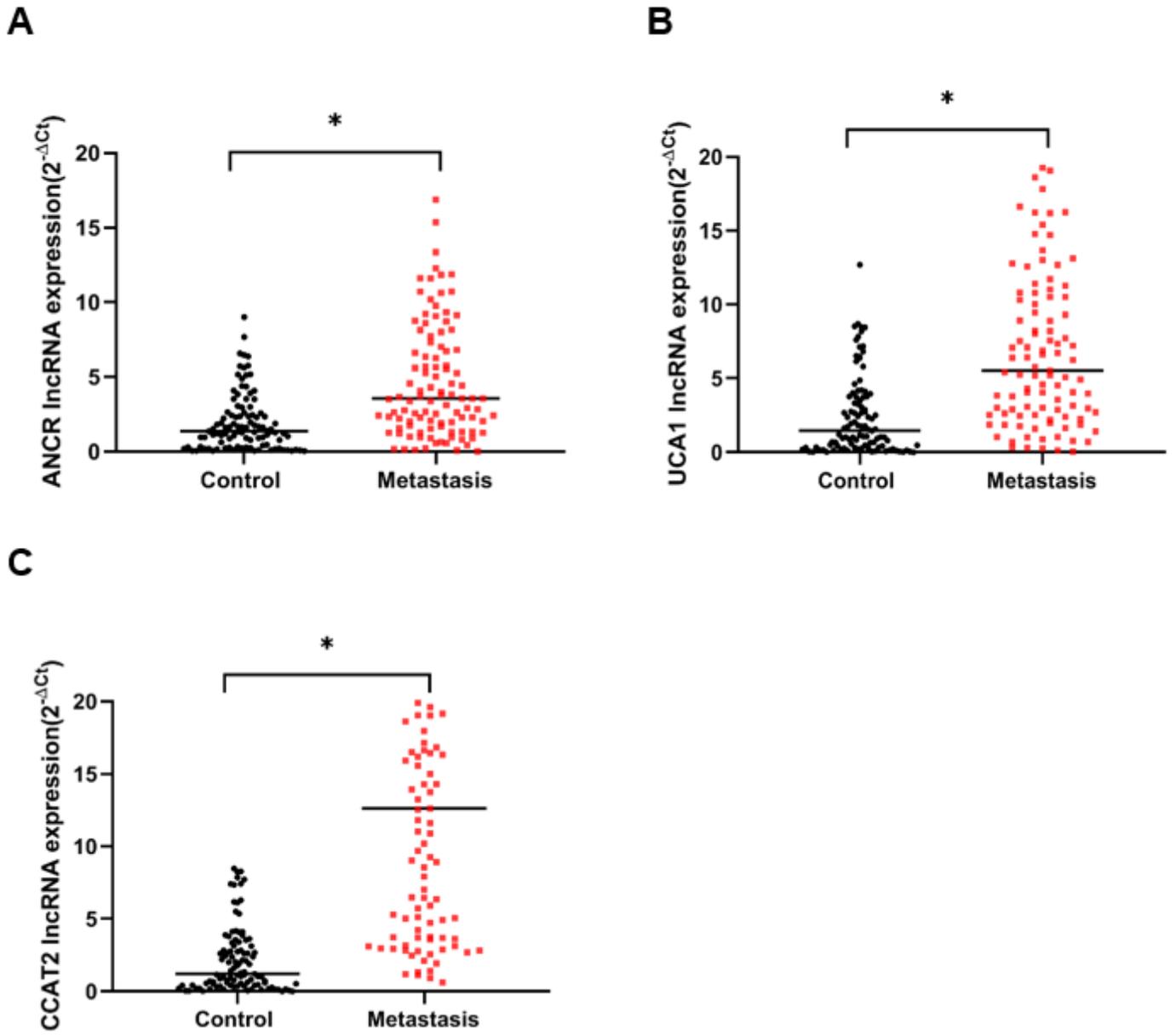
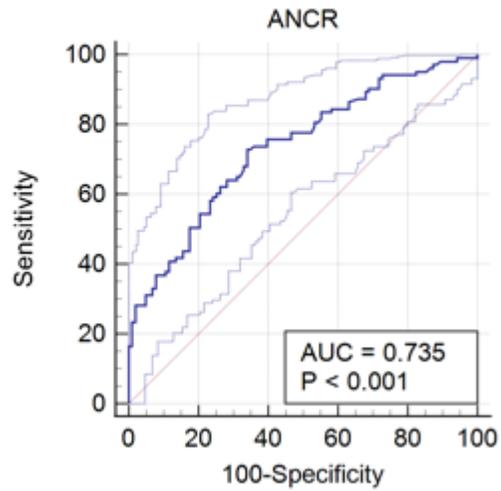
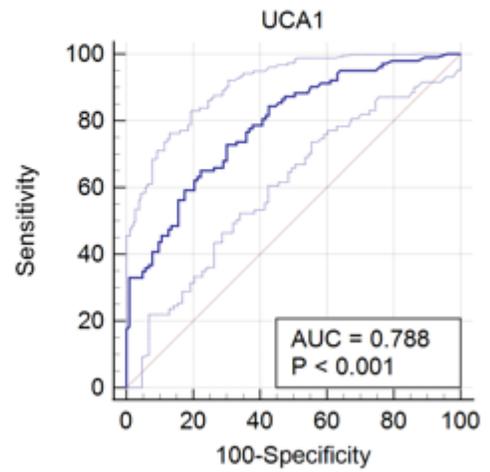
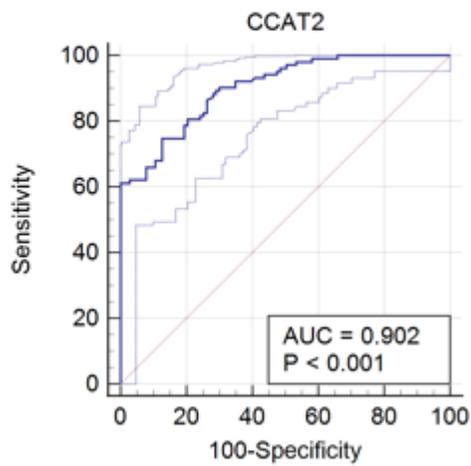


Figure 1

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.

A**B****C****Figure 2**

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

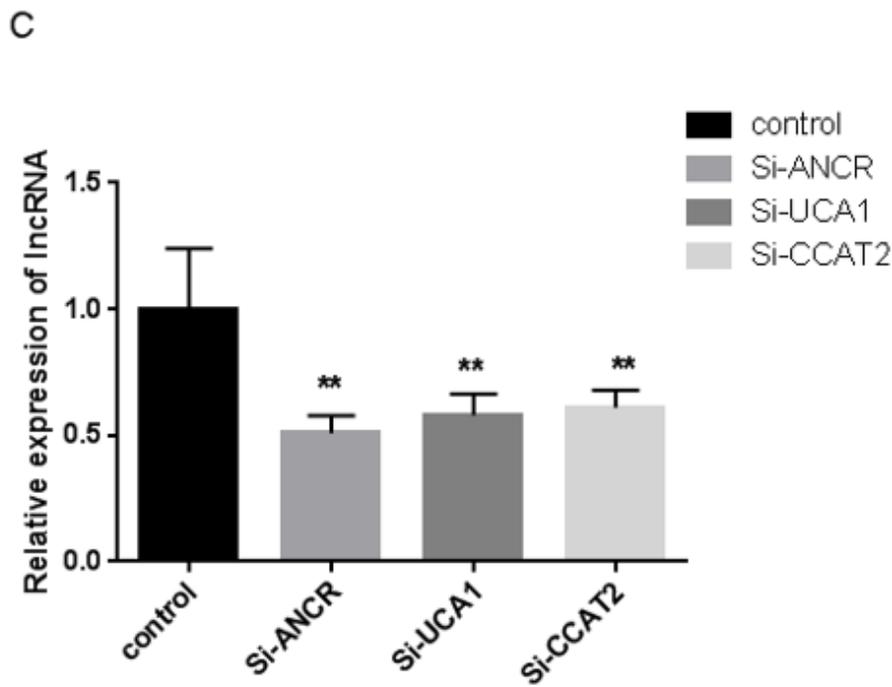
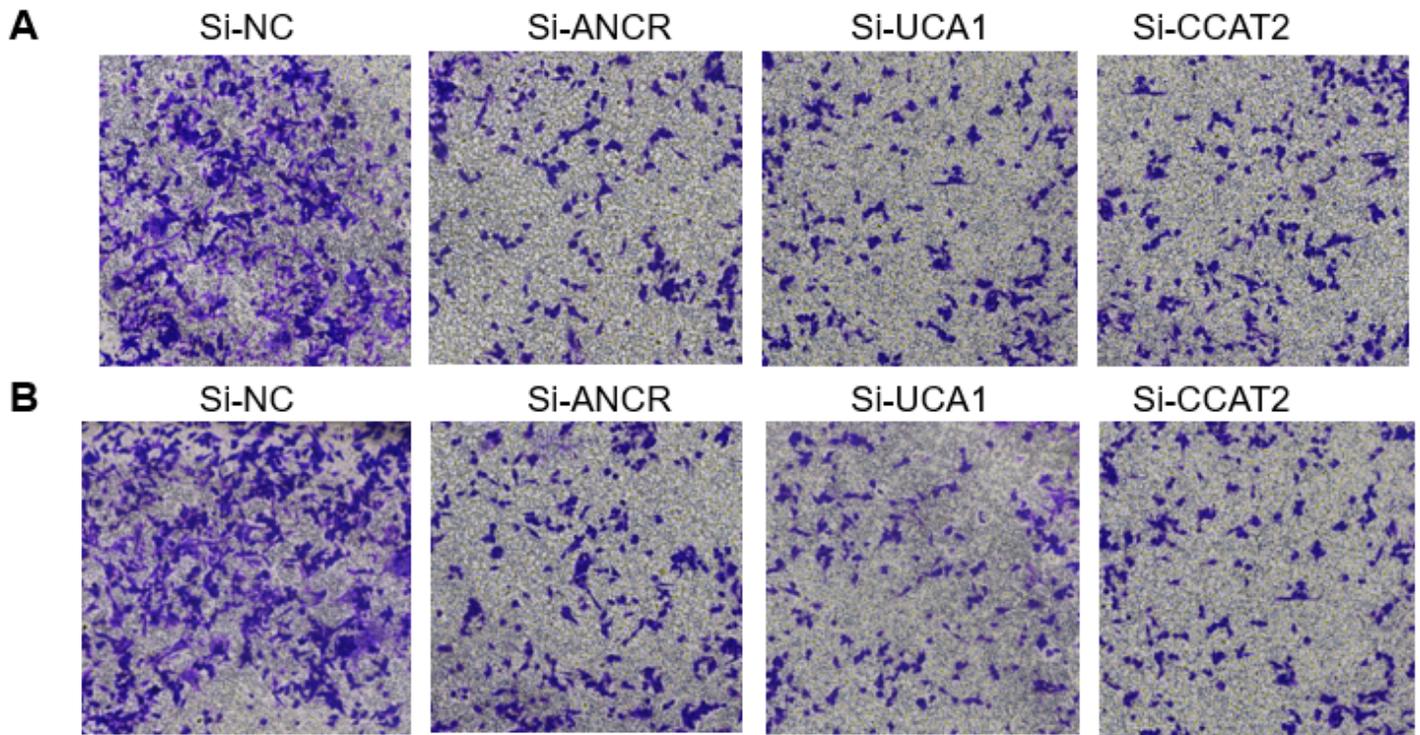


Figure 3

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 \pm SD. **, P < 0.05.versus control.

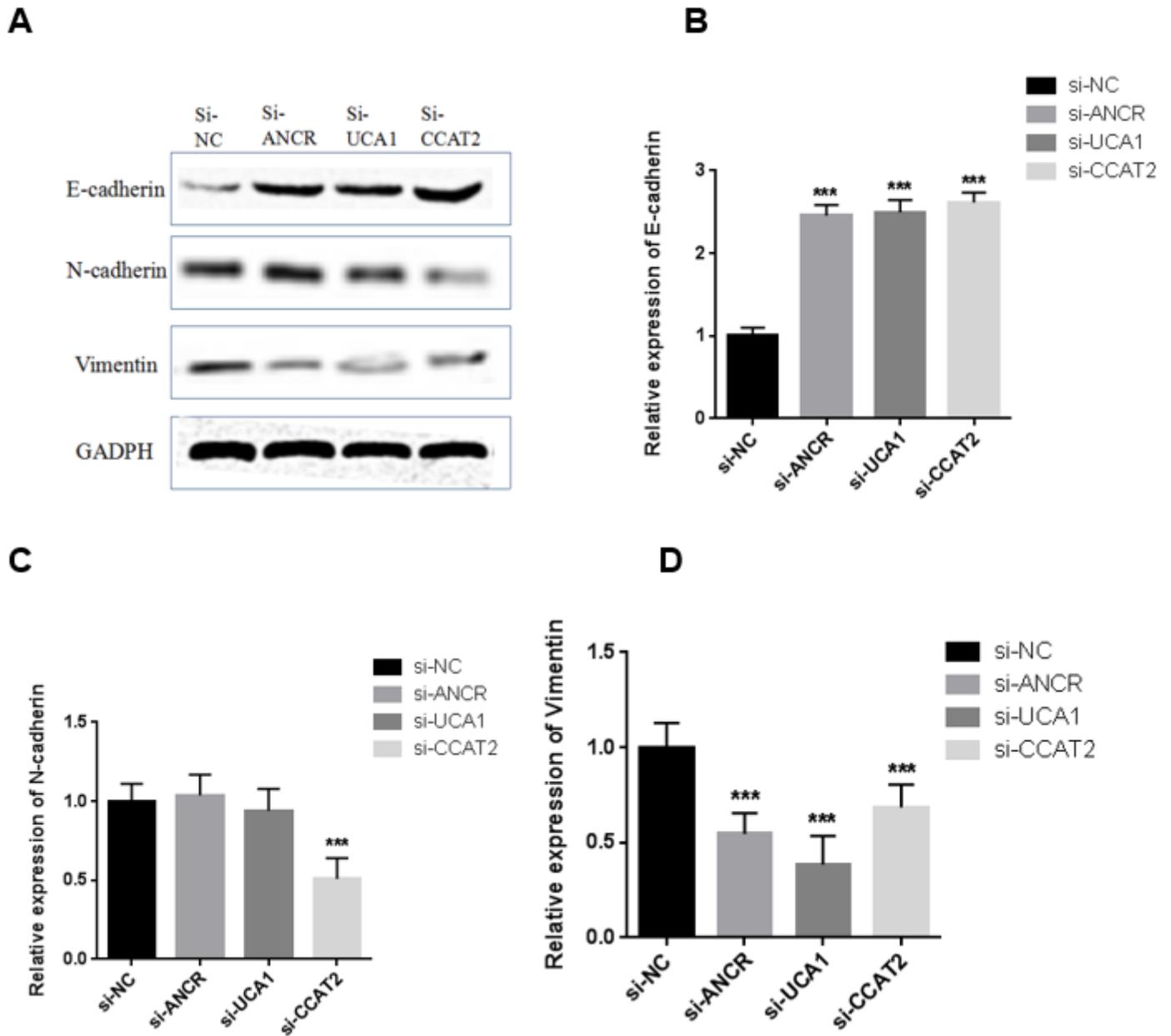


Figure 4

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