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Long non-coding RNA ANCR promotes progression of NSCLC by inhibiting E-Ca expression

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Abstract. – OBJECTIVE: This study aimed to investigate whether long-chain non-coding ANCR is involved in the progression of non-small cell LCa (NSCLC) and its possible molecular mechanisms.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was applied to examine ANCR expression in 48 cases of NSCLC and adjacent normal tissues. In addition, ANCR level in patients of different tumor staging was analyzed. The Kaplan-Meier method was applied to analyze the interplay between ANCR expression and the progn[illegible] patients with NSCLC. Subsequently, q[illegible] was performed to detect ANCR level in L[illegible]ell lines. After knocking down ANCR in A549 [illegible] ANCR and E-Ca mRNA expression were ex[illegible]ined by qRT-PCR, while the expression levels [illegible] epithelial-mesenchymal trans[illegible]MT)-relat[illegible]ed proteins were detected [illegible]blot. At the same time, cell viabilit[illegible] migra[illegible] ability were analyzed through c[illegible]ounting [illegible]CCK-8) and cell wound hea[illegible]say. [illegible] RNA immunoprecipit[illegible]ion [illegible] was performed to verify th[illegible]ding [illegible]R to EZH2. After knocking d[illegible]EZH2 in A[illegible]lls, E-Ca messenger ribo[illegible]acid (mRNA)[illegible]ession was detected [illegible]. Chromatin immunoprecipitation (ChIP) as[illegible]s performed to detect the bi[illegible]ing of EZH2 [illegible] E-Ca promoter region. W[illegible] E-Ca and AN[illegible]re simultaneously k[illegible]ked down in A549 c[illegible]s, Western blot inves[illegible]ion wa[illegible]erformed to examine the expre[illegible]n of E[illegible]elated proteins, while CCK-8 and [illegible]ng as[illegible] were applied to figure ou[illegible]nges i[illegible]l viability and cell mi[illegible]ion ca[illegible]

ULTS: [illegible]evel was conspicuously hig[illegible]n NSC[illegible]ssues than that in normal tiss[illegible] and that in T3 and T4 tumors was also hi[illegible]n T1 and T2. Meanwhile, ANCR e[illegible]ession [illegible]he tissues of patients with lymph [illegible]e metastasis was conspicuously higher than [illegible] without metastasis. Survival analysis re[illegible]ve[illegible]hat the overall survival of patients with NSC[illegible]with high expression of ANCR was conspicuousl[illegible]an patients w[illegible] low expression of [illegible]R. T[illegible]T-PCR study verified that ANCR wa[illegible] highly e[illegible]ed in the LCa cell line A549. [illegible] knocking [illegible]ANCR in A549 cells, AN[illegible] E-Ca mRNA l[illegible]s were found consciously decreased, and so were the expression levels of EM[illegible]-related proteins, as well as cell viability[illegible]d migration ability. The RIP assay result ind[illegible]ted that ANCR can indeed b[illegible] EZH2. [illegible]a mRNA expression was elevated [illegible] knockdown of EZH2 in A549 cells. [illegible]n, the result of CHIP test demonstrated that EZH2 could combine with E-Ca. Simultaneous down-regulation of ANCR and E-Ca in cells could reverse the influence of [illegible]ock[illegible]g down ANCR alone on cell viability and migration ability.

CONCLUSIONS: Long-chain non-coding RNA ANCR was highly expressed in NSCLC tissues and could enhance the viability and malignancy of NSCLC cells by inhibiting the expression of E-Ca, thereby promoting the progression of NSCLC.

Key Words:
NSCLC, ANCR, EZH2, EMT.

Introduction

Lung cancer (LCa) is the main cause of cancer-related morbidity and mortality, accounting for more than a quarter of all cancer diagnoses[1]. Non-small cell lung cancer (NSCLC) is the most important pathological subtype, accounting for about 85% of LCa cases[2,3]. Despite advances in treatment techniques and strategies, the prognosis of this disease remains poor, with a 5-year survival rate of only 15% for NSCLC[4-6].The development of drug resistance, metastasis, and recurrence is the main obstacle to the improvement of patients' survival[7,8].Therefore, it is of

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great significance to further clarify the molecular mechanism of NSCLC progression and provide a scientific basis for new treatment strategies.

Long non-coding RNAs (lncRNAs) are defined as transcriptional classes of over 200 nucleotides lacking protein-coding potential[9]. LncRNAs have been demonstrated as fine tuners and regulators of key biological processes in various cancers, such as cell growth, apoptosis, differentiation, invasion, and metastasis[10,11]. There is evidence that lncRNA is involved in cancer development by altering DNA repair, membrane effusion, drug metabolism, apoptosis, and epithelial-mesenchymal transformation (EMT)[12]. LncRNA ANCR has been reported to inhibit the proliferation, migration, and invasion of osteosarcoma cells by interacting with EZH2 and regulating the expression of p21 and p27[13]. However, the role of LncRNA ANCR in the development of NSCLC is unclear.

EMT is the transformation of cell morphology and function from epithelium to mesenchymal phenotype, and is involved in the early malignant transformation of tumors[14-16]. During EMT, cells lose or redistribute epithelial proteins and a[illegible]re mesenchymal proteins, resulting in loss[illegible] thelial polarity and a high-motion fibrobla[illegible]e-notype[17-19]. Several studies[20-23] have shown[illegible]t EMT plays an important role in many canc[illegible] such as pancreatic cancer, gastric cancer, a[illegible] colorectal cancer. Also, EMT [illegible]ssociated with cancer invasion and me[illegible]sis. I[illegible]s work, LCa cells were used as res[illegible] object[illegible]xplore whether the long non-co[illegible]RNA promote the develop[illegible]t c[illegible] by inhibiting the expression [illegible]Ca.

Pa[illegible]nts [illegible]Methods

Patient[illegible]d Clinical Sa[illegible]

From [illegible]y 2016 to December 2018, 48 patients with [illegible]LC ad[illegible]ed to our hospital underwent su[illegible]rese[illegible] of tu[illegible] tissues and adjacent non-t[illegible]es. A [illegible]f adjacent normal tissues are [illegible]ted a[illegible]st 3 cm from the tumor [illegible]ry. [illegible]ally removed tissue was rapi[illegible] frozen [illegible]quid nitrogen until analysis. All [illegible]48 patients had no history of treatment ar[illegible] other malignancies. This investi[illegible]ion was approved by the Ethics Committee of [illegible]bo Medical Center Eastern Hospital. Signed w[illegible] informed consents were obtained from all parti[illegible]nts before the study.

Cell Culture

HBE, A549, NCI-H1650, and HC[illegible] cells were purchased from the Shan[illegible]ell B[illegible] of the Chinese Academy of Scie[illegible] (Shanghai, China). The cells were culture[illegible]oswell Park Memorial Institute-1640 (RPMI[illegible] Hyclone, South Logan, UT, USA) m[illegible]m co[illegible]10% fetal bovine serum (FBS[illegible]clone, Sout[illegible] UT, USA) and 1% stre[illegible]ycin [illegible]penici[illegible] 37°C, with 5% CO_2, [illegible]ated h[illegible]ity. The cells in the exponential [illegible] p[illegible] were [illegible]sted, plated into appr[illegible]iate [illegible]es, a[illegible]ltured in an incubato[illegible]l the ce[illegible]ity [illegible]es 60%.

Cell Tra[illegible]

LCa [illegible] wer[illegible]d in a cell plate and the transfection of si-A[illegible] si-EZH2, si-E-Ca, and thei[illegible]ve negative c[illegible]s were performed w[illegible]Lipofectamine 2000 (Invitrogen, Carlsbad, [illegible] USA) transfe[illegible]ion reagent when cell density [illegible]ched 60%. A[illegible]48h, cells were collected for [illegible]equent inves[illegible]tions.

RN[illegible]n and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Detection

[illegible]d tissue total RNA were extracted us[illegible]g th[illegible]RIzol (Invitrogen, Carlsbad, CA, USA) method. The RNA purity was measured by an ultraviolet spectrophotometer and was stored at –80°C until the use. The complementary deoxyribonucleic acids (cDNAs) was reverse transcribed, and the SYBR Green method was used for PCR detection. The primer sequences were shown in Table I.

Western Blot

The total protein was extracted from each group and subjected to dodecyl sulfate and sodium salt-polyacrylamide gel (SDS-PAGE) electrophoresis. After transferred to the polyvinylidene difluoride (PVDF) membranes, the immunoblots were blocked for 2 h at room temperature with a specific primary antibody overnight. Then, they were incubated with horseradish peroxidase (HRP)-labeled secondary antibody at room temperature for 2 h. Next, the protein bands were detected using enhanced chemiluminescence (ECL) detection.

Cell Counting Kit-8 (CCK-8) Assay

Differently treated cells were plated in 96-well plates with 5 replicates in each group and cultured for 6, 24, 48, 72, 96 h, respectively. 10 μL of

Table I. Primers used for qRT-PCR.

Gene	Sequence
EZH2	F: 5'-TGCACATCCTGACTTCTGTG-3' R: 5'-AAGGGCATTCACCAACTCC-3'
E-ca	F: 5'-AAAGGCCCATTTCCTAAAAACCT-3' R: 5'-TGCGTTCTCTATCCAGAGGCT-3'
ANCR	F: 5'-GACATTTCCTGAGTCGTCTTC[illegible]CGGAC-3' R: 5'-TAGTGCGATTTAGAGCTGT[illegible]AGTTT[illegible]3'
GAPDH	F: 5'-CGGAGTCAACGGATTTG[illegible]GT-3' R: 5'-GGGAAGGATCTGTCTC[illegible]C-3'

CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added to each well, and cells were further incubated at 37°C for 1 h. The OD values were measured at 450 nm.

Cell Wound Healing Assay

The cells in log phase were collected and seeded in 24-well plates at a density of approximately 5×10^5 cells per well. Then, they were incubated overnight. The tip of the liquid pipe was drawn across the bottom of the well. Subsequently, the cells at the scratches were was[illegible] as much as possible with phosphate-buffere[illegible] (PBS), cultured in a serum-free mediu[illegible]nd photographed under a microscope at 0 and [illegible] respectively. The scratch distance in each gr[illegible] was measured, and the average cell mobility w[illegible] calculated by averaging. Cell [illegible]= (initial scratch average distance - [illegible]age [illegible]ch distance measured) / initial s[illegible]n avera[illegible]istance × 100%.

RNA Binding P[illegible]in Immunoprec[illegible] (RIP) Assay

We perfor[illegible] RI[illegible]iments according to the manufa[illegible]turer's instr[illegible] of Magna RIP RNA Bi[illegible] Protein Imm[illegible]cipitation Kit (Perkin[illegible]er, Waltham, MA, [illegible]SA). After the cell l[illegible]e was c[illegible]ed, the magnetic beads were pre[illegible] and [illegible]y resu[illegible]ended in Wash Buffer and pr[illegible]e. RNA[illegible]ding protein immunoprecipitat[illegible]s the[illegible]erformed. RNA purifications ca[illegible]y phenol, chloroform, Salt Solu[illegible] I, Salt [illegible]ion II, Precipitate Enhancer, abso[illegible]ethanol (no RNAse), dissolved in 10-20 ul[illegible]carbonate (DEPC) water (Beyo-[illegible], Shanghai, China), and stored at –80°C. The [illegible]ession of ANCR in co-precipitated EZH2 p[illegible] and IgG protein precipitate was detected by q[illegible]PCR.

Chromatin [illegible]munoprecip[illegible] (ChIP)

Chrom[illegible]unoprecipitat[illegible] was performed [illegible]g a[illegible] ChIP A/G One-Color Chromatin Immunop[illegible]itation Kit (Millipore, Bill[illegible]MA, USA) ac[illegible]g to the manufacturer's instructions. Chromatin immunoprecipitated DNA was el[illegible]ed, reverse X-linked, purified, [illegible] analyzed by [illegible]T-PCR.

S[illegible]ical An[illegible]sis

A[illegible] statistically analyzed by Statistical Product and Service Solutions (SPSS) 16.0 (SPSS, Chicago, IL, USA) statistical software. The data of each group were expressed [illegible]± standard deviation ($\bar{x} \pm s$). The independent sample *t*-test was used to compare the quantitative data of the two groups. The cumulative survival rate was assessed by the Kaplan-Meier method, and the difference was determined by the log-rank test. $p<0.05$ was considered significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

ANCR is Highly Expressed in NSCLC Tissues and Negatively Correlated with Prognosis

To explore the relation between the expression of ANCR and the development of NSCLC, we used qRT-PCR to detect the expression of ANCR in NSCLC tissues and adjacent normal lung tissues. The experimental results show that the expression level of ANCR in NSCLC tissues was conspicuously higher than normal lung tissues (Figure 1A). We, then, performed a paired analysis of the tissue samples and found that the expression levels of ANCR in T3 and T4 tumors were conspicuously higher than those in T1 and T2 (Figure 1B). At the same time, we found that ANCR was higher in metastasis group than in

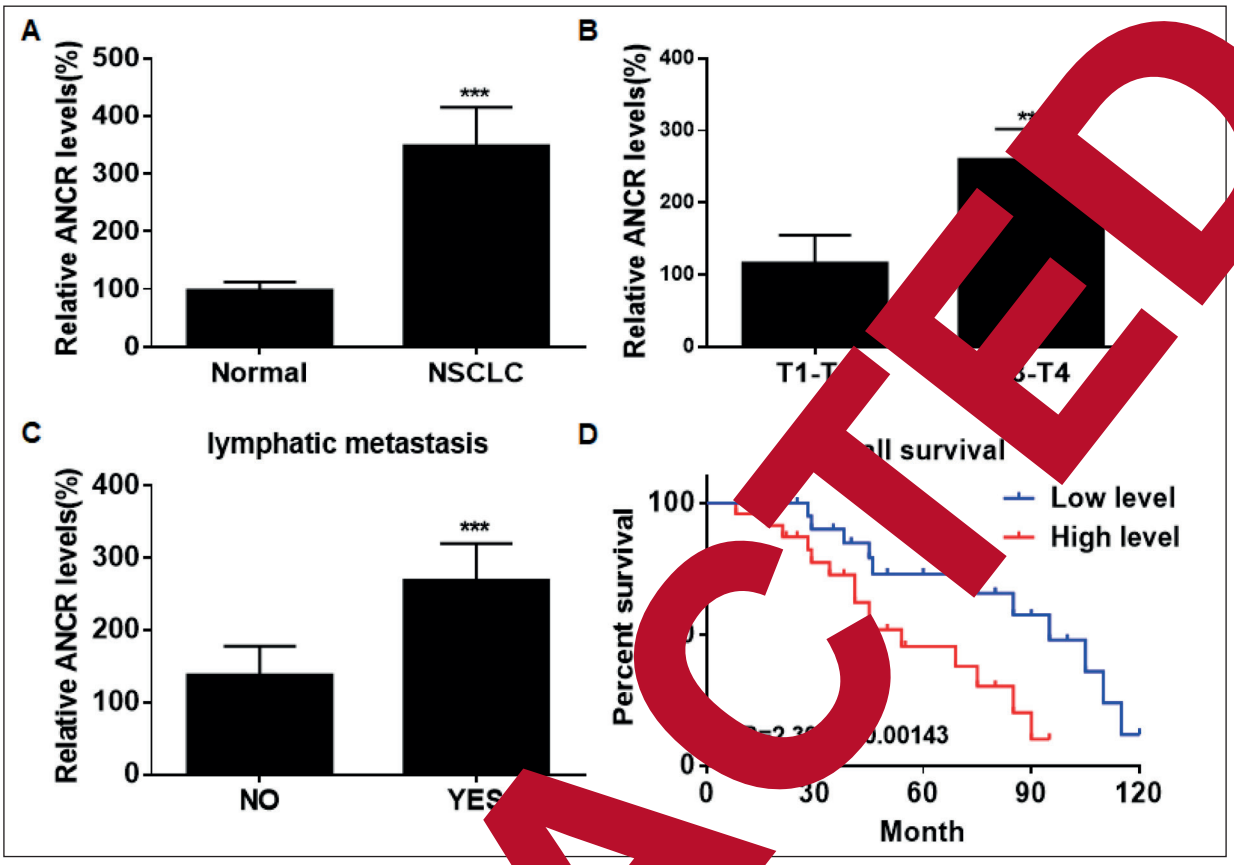


Figure 1. ANCR was found highly expressed in nor l ce g can r tissues and negatively correlated with prognosis of patients with NSCLC. **A,** ANCR was highly expresse mall cell lung cancer tissues. **B,** Expression levels of ANCR in the T1 and T2 phases were higher than those in the T4 phases. **C,** Expression of ANCR in the metastatic group was higher than that in the non-meta p. **D,** Ove rvival rate of patients with high expression of ANCR was significantly lower than that of pati xpression.

NSCLC group (Figure Kaplan ysis showed that the era of patients with NSCLC in hig pressio of ANCR was conspicuous wer than th patients with low expre ure 1D). The ove results indicate at g-chain non-coding RNA ANC was highly essed in NSCLC tissues a gatively correla th prognosis.

Low pressi of ANCR blocks EMT ar lign y in NSCLC

To he role lncRN ANCR in the d velopi f NSC , we used qRT-PCR to e the of ANCR in lung normal cell E and L cells A549, NCI-H1650, and HC qRT-PCR results showed that ANCR w essed in LCa cells, and had the est expression level in A549 cell line. There- we chose A549 cells for subsequent inves- g (Figure 2A). Subsequently, we knocked dow e expression of ANCR in A549 cells (Figure 2B). The results of Western blots showed that the level of E-Ca protein in epithelial cells increased after knockdown of ANCR, and the expression of N-ca, and Vimentin proteins in mesenchymal cells decreased (Figure 2C). The results of the CCK-8 experiment evidenced that the cell viability of A549 LCa cells was conspicuously reduced after knocking down ANCR (Figure 2D). In addition, the results of the cell scratch test highlighted that the migration ability of A549 LCa cells was conspicuously reduced after interfering with ANCR (Figure 2E). The above results indicated that low expression of ANCR can inhibit the proliferation and malignancy of NSCLC.

ANCR Binds to EZH2 to Regulate the Expression of E-Ca

To further explore the molecular mechanism of lncRNA ANCR in the development of NSCLC, qRT-PCR results showed that E-Ca mRNA expression was elevated after interference with AN-

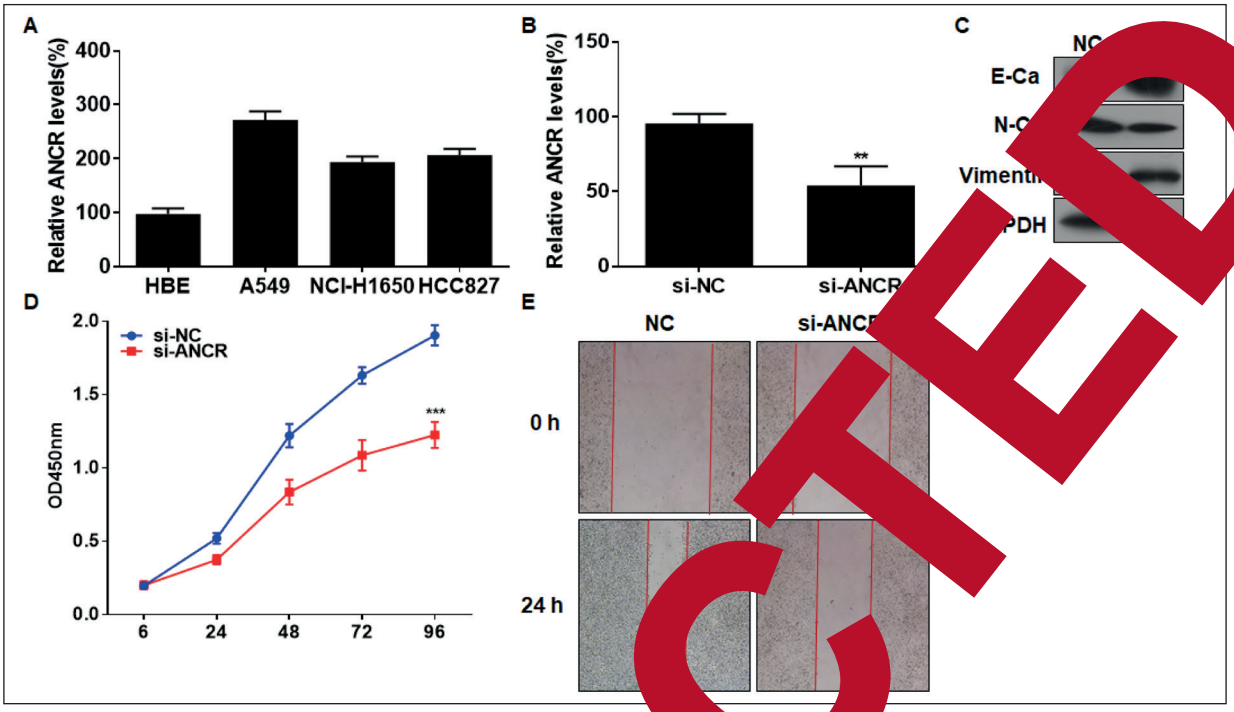


Figure 2. Interfering with the expression of ANCR inhibited E[illegible] non-small[illegible] lung cancer, and thus reduced its malignancy. **A,** Expression of ANCR in normal cell HBE and non-small[illegible] cell lines including A549, NCI-H1650, and HCC827. **B,** Interference sequences were constr[illegible] to knock down the expression level of ANCR. **C,** Results of Western blots showed that the level of E-ca protein in epi[illegible] increased in A549 cells after interfering with ANCR, and the expression levels of N-ca and Vimentin pro[illegible] lls were decreased. **D,** CCK-8 results indicated that interference with ANCR significantly inhibited [illegible] viabil[illegible] ells. **E,** Cell wound healing assay revealed that interference with ANCR significantly inhibited the m[illegible] y of A549 cells (magnification: 40×).

CR in A549 cells (Figure 3A)[illegible] me time, the RIP research results s[illegible] sted [illegible] ANCR can be combined with EZ[illegible] Figure [illegible] Subsequently, we constructed [illegible] rfere[illegible] of EZH2 and knocked [illegible] A549 cells (Figure 3C), and q[illegible] CR re[illegible] tected that E-Ca mRNA exp[illegible] n levels inc[illegible] (Figure 3D). The CHI[illegible] owed that E[illegible] could be combined [illegible] E[illegible] fter interfering with ANCR, th[illegible] combination [illegible] ZH2 with E-Ca decrease[illegible] spicuously (Fi[illegible] E). The above results [illegible] cated that ANCR co[illegible] d bind to EZH2 in LC[illegible] lls to [illegible] ate the expression of E-Ca.

ANC[illegible] tes th[illegible] alignancy of NSCLC [illegible] hibit[illegible] the Expression [illegible] Ca

T[illegible] bserve t[illegible] ble of ANCR and E-Ca in tum[illegible] rogression, we constructed an interfering se[illegible] fere with the expression level of [illegible] in A549 cells (Figure 4A). Subsequently, we [illegible] fered with E-Ca after ANCR knockdown in A[illegible] lls. The results of Western blots showed inter[illegible] nce with E-Ca reversed the level of E-Ca protein in epithelial cells in A549 cells caused by ANCR silencing. Besides, the expression levels of mesenchymal cell markers, N-ca and Vimentin proteins decreased (Figure 4B). CCK-8 investigations showed interference with E-Ca reversed the decrease in A549 cell viability caused by interference with ANCR (Figure 4C). Meanwhile, cell scratch test results revealed that interference with E-Ca reversed the inhibition effect of ANCR silencing on cell migration (Figure 4D). The above results indicated that ANCR promoted the proliferation and malignancy of NSCLC by inhibiting the expression of E-Ca.

Discussion

NSCLC is the most common type of LCa and the leading cause of cancer death worldwide[24]. However, there has been relatively little research on the potential importance of long non-coding RNAs in determining their biology and outcomes. We found that ANCR was highly expressed in NSCLC tissues, and ANCR expression in T3 and

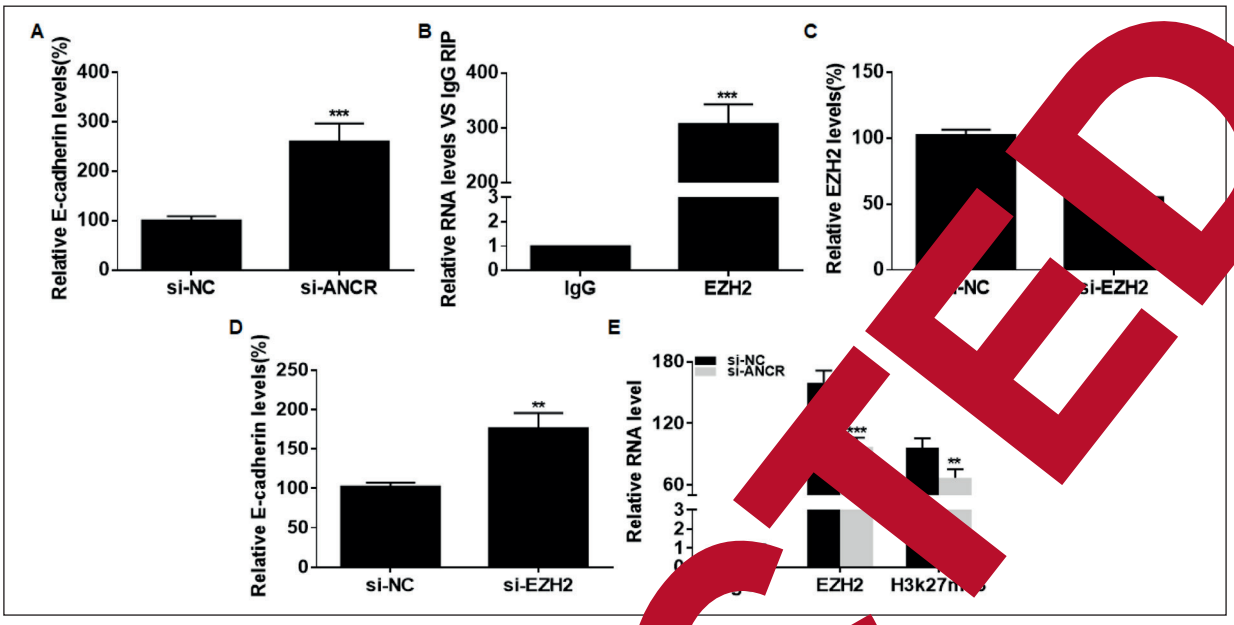
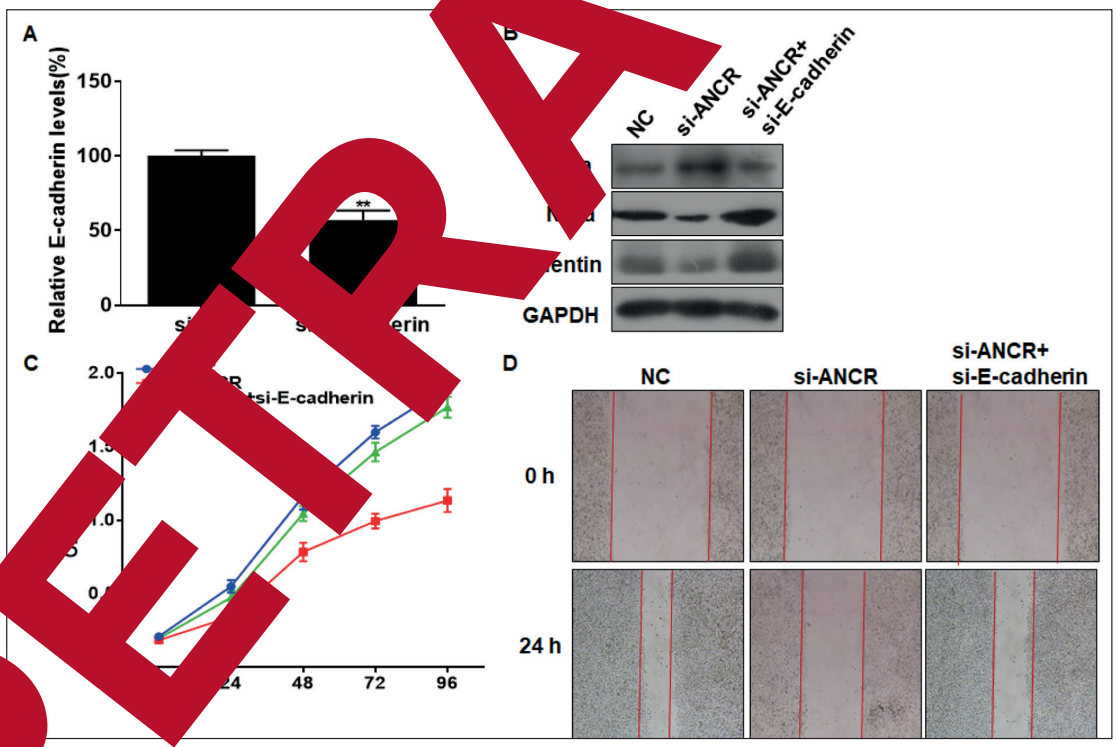


Figure 3. ANCR could combine with EZH2 to regulate E-ca exp on. **A,** E-ca mR expression level was increased after ANCR was inhibited in A549 cells. **B,** RIP experiment results sh that ANCR can bine with EZH2. **C,** In A549 cells, interference sequences were constructed to knock down the expres vel of EZH2 fter interfering with the expression level of EZH2 in A549 cells, qRT-PCR results showed that the mRN sion lev -ca increased. **E,** CHIP test showed that EZH2 could combine with E-ca; however, interfering with ANCR bility.



Fi omoted the malignancy of non-small cell lung cancer by inhibiting the expression of E-ca. **A,** Interfering nces were constructed in A549 cells to down-regulate the expression level of E-ca. **B,** Western blots showed that ference with E-ca could reverse the increased protein level of E-Ca and decreased protein levels of N-ca and Vimentin by downregulation of ANCR. **C,** CCK8 experiments showed that interference with E-ca reversed the decrease in A549 ce ty caused by interference with ANCR. **D,** Results of cell wound healing assay showed that interference with E-ca revers he weakened migration ability of A549 cells induced by interference of ANCR (magnification: 40×).

T4 LCa tissues was conspicuously higher than that in T1 and T2 LCa tissues. Survival analysis disclosed that the overall survival time of patients with high ANCR expression was conspicuously lower than that of patients with low ANCR expression, suggesting that ANCR plays an important role in the development of NSCLC.

LncRNAs have been reported to be associated with a survival rate of LCa patients, which may be a biomarker for prognosis of NSCLC patients[25]. LncRNA regulates gene expression by binding to transcription factors, RNA splicing, DNA, and histone modifications[26-28]. EZH2 (enhancer of zeste homolog 2), a 751-amino acid histone-lysine methyltransferase, is located on human chromosome 7q35[29]. EZH2 is an important component of PRC2's catalytic complex, which catalyzes trimethylation of lysine 27 of histone 3 and mediates target gene silencing[30-32]. Sun et al[33] have found that lncRNA hoxa11-as can combine with PRC2 to promote the proliferation and invasion of gastric cancer. In this report, it was found that low ANCR expression in LCa cells could inhibit the EMT level and malignancy degree of LCa cells, and the combination of ANCR and EZH2 silenced the E-Ca gene.

Calcium-adhesive proteins (cagps) are calcium-dependent cell adhesion molecules that are essential for the formation of tight connections between cells[34,35]. E-Cadherin (E-Cad) is the most important one in epithelial cells. Cadherin forms complexes with cytoplasmic proteins, and these molecular complexes, together with other cytoskeletal components, form intercellular adhesion junctions[22,36]. E-Cad changes in the loss of E-Cadherin expression in epithelial cells[37]. In this research, it was found that after the knockdown of ANCR, epithelial cells in A549 showed increased levels of Pet[illegible]t E-Ca protein, decreased expression levels of mesenchymal markers n-ca, and vimentin protein. Furthermore, knockdown of ANCR reversed the EMT process of cancer cells and conspicuously decreased cell viability and migration ability of A549 cells. The work showed that ANCR induced and promoted the proliferation and malignancy of NSCLC by inhibiting the expression of E-Ca

Conclusions

We demonstrated that long non-coding RNA ANCR could promote the development of NSCLC by binding to EZH2 and downregulating the expression of E-Ca. In addition, ANCR could be used as a potential prognostic marker and therapeutic target for patients with NSCLC.

Conflict of Interest

The Authors declare that they have no conflicts of interest.

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