# Long Noncoding RNA NEAT1 Promotes Growth and Metastasis of Cholangiocarcinoma Cells

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Long noncoding RNAs (lncRNAs) are known to play important roles in cancers. However, little is known about lncRNAs in cholangiocarcinoma (CCA), a cholangiocyte malignancy with poor prognosis. We investigated the role of nuclear paraspeckle assembly transcript 1 (NEAT1) lncRNA in promoting CCA. qRT-PCR analysis of patient samples showed that NEAT1 expression was higher in CCA tumors than in matched adjacent nontumor tissue. NEAT1 levels were also higher in CCA cell lines than in a normal biliary epithelium cell line (HIBEpic). NEAT1 knockdown in CCA cell lines using shNEAT1 reduced cell proliferation and colony formation in CCK-8 and colony formation assays, respectively. CCA cells transfected with shNEAT1 also exhibited reduced metastasis and invasiveness in Transwell assays. NEAT1 knockdown cells produced smaller tumors, demonstrating that NEAT1 promotes tumor growth in vivo. Silencing of NEAT1 increased E-cadherin expression in vitro, and E-cadherin expression was inversely correlated with NEAT1 expression in CCA tissue samples. RIP and ChIP assays suggest that NEAT1 is recruited to the E-cadherin promoter by EZH2 (enhancer of zeste homolog 2), where it represses E-cadherin expression. These findings indicate that NEAT1 exerts oncogenic effects in CCA. We postulate that NEAT1 is a potentially useful diagnostic and therapeutic target for CCA.

Key words: Long noncoding RNAs (lncRNAs); Cholangiocarcinoma (CCA); E-cadherin;
Nuclear paraspeckle assembly transcript 1 (NEAT1); Enhancer of zeste homolog 2 (EZH2)

## INTRODUCTION

Cholangiocarcinoma (CCA) is one of the most offensive and lethal tumors originating from malignant conversion of cholangiocytes and epithelial cells lining the intrahepatic and extrahepatic biliary ducts[1]. CCA is an aggressive cancer with a median survival of fewer than 24 months after diagnosis. Curative surgery is suggested only for early stage patients and is not available for advanced stage patients[2]. Despite the numerous improvements of surgical resection and standard chemotherapeutic agents, patients with CCA have inferior overall survival rates and poor prognosis, indicating that new and practical strategies are required to identify novel therapeutic targets by deciphering the critical molecular mechanisms regulating CCA to improve patient survival times.

Long noncoding RNAs (lncRNAs), which are more than 200 nucleotides in length, are key regulators of cellular transcription. Dysregulated expressions of lncRNAs have been reported in many types of cancers, indicating that they may play significant roles in tumorigenesis[3–5]. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) has been identified as a prognostic marker for lung cancer metastasis and acts as a regulator of gene expression[6]. Prostate-specific transcript 1 (PCGEM1) is classified as a novel, highly prostate tissue-specific gene and supports prostate cancer progression[7]. Recently, the mechanisms and functions of various lncRNAs, such as HEIH, HULC, and HOTAIR, were revealed in hepatic carcinoma, the most frequent hepatic malignancy, and these findings led to the construct of coordination networks and extended our knowledge concerning tumorigenesis in hepatic carcinoma[8–10]. Similarly, we hypothesized the existence of dysregulated lncRNAs that would promote CCA by regulating the key signaling pathways. Hence, identification of cancer-associated lncRNAs and their molecular and biological functions in CCA is important.

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One of the most profoundly regulated lncRNAs in the current pan-genomic datasets is nuclear paraspeckle assembly transcript 1 (NEAT1). NEAT1 is transcribed from the familial tumor syndrome multiple endocrine neoplasia (MEN) type 1 locus at 11q13.1. It performs as a transcriptional regulator of some genes implicated in cancer progression[11]. NEAT1 was found to be a diagnostic and prognostic biomarker in several cancers[12]. A study also showed that NEAT1 was identified as a critical modulator of prostate cancer by interacting with estrogen receptor α[13]. In addition, NEAT1 enhances non-small cell lung cancer (NSCLC) malignant progression and promotes migration and invasion in gastric cancers[14,15]. However, the functional role and underlying mechanism of NEAT1 in CCA are still elusive.

Here we illustrate a novel mechanism by which lncRNA plays oncogenic roles through modulating the status of its interaction protein. First, we verified the remarkably increased expression levels of NEAT1 in CCA tissues compared to their adjacent noncancerous tissues. NEAT1 could improve cell proliferation, migration, and invasion in vitro and in vivo. Furthermore, silencing of NEAT1 increased E-cadherin expression in vitro, and E-cadherin expression was inversely associated with NEAT1 expression in CCA tissue samples. RNA immunoprecipitation (RIP) and chromatin immunoprecipitation (ChIP) assays suggest that NEAT1 is recruited to the E-cadherin promoter by enhancer of zeste homolog 2 (EZH2), where it represses E-cadherin expression. Our findings may contribute potential biomarkers for CCA progression and potential therapeutic targets for the disease.

## MATERIALS AND METHODS

### Patients and Samples

CCA tissues and matched adjacent normal bile duct tissues were obtained from 33 patients who underwent surgery between January 2013 and November 2016 in Zhongshan Hospital, Dalian, P.R. China. All samples were processed by two professional pathologists. The fresh tissue specimens were snap frozen in liquid nitrogen and then stored at −80°C prior to RNA isolation. There was no preoperative treatment prior to surgery. All patients signed informed consent before surgery. This study was approved by the Human Ethics Committee of Zhongshan Hospital at Dalian University (P.R. China) and was carried out in accordance with the Declaration of Helsinki.

### Cell Lines and Cultures

The human CCA cell lines (QBC939, RBE, HuCCT1, and TFK1) and the normal biliary epithelium cell line HIBEpic were grown in RPMI-1640 (Gibco, Carlsbad, CA, USA) medium supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin

(Beijing Solarbio Science & Technology Co., Beijing, P.R. China) at 37°C and 5% $CO_2$.

### Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was extracted from CCA cells using a TRIzol kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using 2 μg of the total RNA according to the instructions of the reverse transcriptase kit (Takara Bio, Inc., Dalian, P.R. China). The cDNA samples (2 μl) were subjected to qRT-PCR using a SYBR® Premix Ex Taq kit (Takara Bio) for 40 cycles. The following were the primers used: NEAT1, 5′-ATGCCACAACGCAGATTGAT-3′ (forward) and 5′-CGAGAAACGCACAAGAAGG-3′ (reverse); EZH2, 5′-CCGCAAGGGTAACAAAAT-3′ (forward) and 5′-GGTAGCAGATGTCAAGGGA-3′ (reverse); E-cadherin, 5′-TGTCCGCCCCGACTTGTCTCTC-3′ (forward) and 5′-GTCCTCTGGCCCCAGCCTCTCT-3′ (reverse); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGCACCACCAACTGCTTAGC-3′ (forward) and 5′-GGCATGCACTGTGGTCATGAG-3′ (reverse). The cycle threshold (Ct) of different genes was first normalized to GAPDH for the same sample, and fold changes were calculated through relative quantification ($2^{-\Delta\Delta Ct}$).

### Knockdown of NEAT1 by Short Hairpin RNA (shRNA) Transfection

The *NEAT1* knockdown (*NEAT1-KD*) plasmid and the respective nontargeting sequence (negative control, *NEAT1-NC*) were synthesized (GeneChem, Shanghai, P.R. China). Cells were transfected through the use of Opti-MEM and Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions when cells were at 50%–70% confluence. The applicable stably transfected cell lines were established by selection with G418 screening. The expression of NEAT1 was confirmed by qRT-PCR.

### RNA Interference (RNAi)

Cells were plated in six-well plates and transfected with 3 μl of EZH2 RNAi in the presence of 4 μl of RNAiMAX according to the manufacturer's instructions. One siRNA for EZH2 and one negative control siRNA were synthesized by Genepharm Technologies (Shanghai, P.R. China). The EZH2 RNAi sequences were as follows: EZH2-si, 5′-GGAUGGUACUUUCAUUGAATT-3′; scramble sequence, 5′-UUCUCCGAACGUGUCACGUTT-3′. Gene silencing effects were confirmed by qRT-PCR analysis at 48 h posttransfection.

### Cell Proliferation Assay

A cell counting kit-8 (CCK-8; Dojindo Laboratories Co. Ltd, Kumamoto, Japan) was used as a colorimetric

assay to assess cell viability. Cells were plated in 96-well plates in triplicate before measurement. CCK-8 solution (10 μl) was added into each well and incubated for 2 h at 37°C. Absorbance was calculated on a Gen5 microplate reader (BioTek, Winooski, VT, USA) at 450 nm. The tests were repeated at least three times.

### Colony Formation Assay

Cells (500 cells/well) were seeded into six-well plates and cultured for 2 weeks. After fixation in 4% paraformaldehyde for 10 min, cells were stained with 1% crystal violet. Colonies with diameters greater than 100 μm were counted, and experiments were run independently in triplicate.

### Western Blotting

Cellular protein was extracted from tissue samples and cells using RIPA lysis buffer. The protein concentration was measured using the BCA protein assay kit (Beyotime Biotechnology, P.R. China). A standard Western blot analysis of the lysates was performed with a primary monoclonal antibody to E-cadherin (1:200; Abcam, Cambridge, UK), N-cadherin (1:200; Abcam), vimentin (1:200; Abcam), and an antibody to GAPDH (1:1,000; Abcam) overnight at 4°C. After washing with Tris-buffered saline Tween 20 (TBST), the membranes were incubated with a secondary antibody against mouse immunoglobulin G (IgG). The membranes were washed, and the proteins were detected using enhanced chemiluminescence (ECL; Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

### In Vitro Migration and Invasion Assays

The migration and invasion assays were conducted in a modified 24-well Boyden chamber using an uncoated membrane and a membrane coated with Matrigel (BD Biosciences, San Jose, CA). Briefly, cells ($5\times10^4$) were seeded into the upper chambers, and 10% FBS was filled in the lower wells. After 24 h of incubation at 37°C in a $CO_2$ incubator, the nonmigratory and noninvasive cells in the upper chamber of the filter were removed with a cotton swab. The average numbers of migratory and invasive tumor cells were counted from five randomly selected 20× fields for each experiment.

### RNA-Binding Protein Immunoprecipitation (RIP)

RIP kits were purchased from Millipore (Darmstadt, Germany). RIP was performed according to the manufacturer's protocol. Briefly, cells were harvested and lysed in RIP lysis buffer. NEAT1 was immunoprecipitated with an EZH2 antibody (Cell Signaling Technology, Danvers, MA, USA). The magnetic bead bound complexes were immobilized with a magnet, and unbound materials were washed off. Finally, RNAs were extracted and stored at −80°C for further qRT-PCR analysis.

### ChIP Assay

ChIP assays were performed on cell line DNA using an Imprint Chromatin Immunoprecipitation Kit according to the manufacturer's instructions (Millipore, Boston, MA, USA). In brief, RBE cells ($5\times10^6$) were treated with 1% formaldehyde for 10 min for cross-linking and then quenched by the addition of 0.125 M glycine. The cells were scraped with PBS solution and gathered after centrifugation at $800\times g$ for 5 min at 4°C. The cross-linked cells were resuspended in sodium dodecyl sulfate (SDS) lysis buffer containing protease inhibitor cocktail II, and the soluble chromatin was sheared to fragment DNA by nuclear lysis buffer. The fragmented chromatin samples were aliquoted as genomic input DNA or immunoprecipitated with EZH2 (Cell Signaling Technology) and H3K27me3 antibodies (Cell Signaling Technology) or IgG (Cell Signaling Technology) and incubated at 4°C with rotation overnight. Immunocomplexes, collected by a magnetic separator, were washed and eluted with ChIP elution buffer. DNA was purified on spin columns. The ChIP products and genomic input DNA were analyzed by RT-PCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The following were the three primer pairs of E-cadherin used for the ChIP assays: 1, 5′-TGTCCGCCCCGACTTGTCTCTC-3′ (forward) and 5′-GTCCTCTGGCCCCAGCCTCTCT-3′ (reverse); 2, 5′-AGACCCCATCTCCAAAACGAACAA A-3′ (forward) and 5′-GCATAGACGCGGTGACCCT CTAGCC-3′ (reverse); 3, 5′-TGTCCGCCCCGACTTG TCTCTC-3′ (forward) and 5′-CGGTCCTCTGGCCCC AGCCTCT-3′ (reverse).

### Animal Experiments

All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Zhongshan Hospital affiliated with Dalian University. All animal experimental procedures were in accordance with the institutional ethical guidelines for animal experiments. All surgical procedures were conducted under sodium pentobarbital anesthesia and minimal animal suffering. For the tumorigenicity assay, cells ($2\times10^6$ cells, suspended in 100 μl of RPMI-1640 without FBS) were subcutaneously injected into the upper right flank of nude mice (4–6 weeks old, BALB/c/nu, female). Tumor sizes were measured by a vernier caliper. Mice were sacrificed 6 weeks after inoculation, and the tumor tissues were harvested, weighed, imaged, embedded in 10% paraffin, and subjected to immunohistochemistry (IHC) staining.

### Statistical Analyses

The results of the continuous variables are presented as mean±SD unless otherwise stated. Treatment groups were compared using independent sample t-tests. Pairwise

multiple comparisons were performed by one-way ANOVA (two sided). A value of $p < 0.05$ was considered statistically significant. All analyses were performed using IBM SPSS statistics software version 19.0 (Chicago, IL, USA).

## RESULTS

### Aberrant Overexpression of NEAT1 in Human Cholangiocarcinoma Tissues

The levels of NEAT1 expression in all four CCA cell lines (HuCCT1, TFK1, RBE, and QBC939) were significantly elevated compared with NEAT1 expression of the normal biliary epithelium cell line (HIBEpic) (Fig. 1A). As NEAT1 was upregulated in CCA cell lines, our interest focused on the NEAT1 expression pattern in CCA tissues. We examined the expression levels of NEAT1 in 33 CCA and adjacent normal patient tissues by qRT-PCR and observed that its expression was significantly upregulated in the CCA tissues ($p < 0.05$) (Fig. 1B). These data demonstrated that NEAT1 had an oncogenic role in CCA.

### Knockdown of NEAT1 Inhibits CCA Cell Proliferation In Vitro

To further examine the role of NEAT1 in CCA progression, we knocked down NEAT1 expression in RBE and QBC939 cells by stable transfection of either shControl or shNEAT1 as shown in Figure 2A. We then performed cell proliferation assays using the CCK-8 assay kit and observed that knockdown of NEAT1 expression significantly suppressed cell growth in RBE and QBC939 cells compared to the controls (Fig. 2B). In addition, downregulation of NEAT1 significantly inhibited colony formation

in RBE and QBC939 cells (Fig. 2C). These data suggested that NEAT1 played a critical role in CCA cell proliferation in vitro.

### Knockdown of NEAT1 Inhibits CCA Cell Migration and Invasion In Vitro

The most important traits of cancers are unrestricted cancer cell growth and migration. Therefore, we evaluated the consequences of NEAT1 knockdown on CCA cell motility by Transwell assays. We observed that knockdown of NEAT1 markedly inhibited the migration of RBE and QBC939 cells (Fig. 2D and E). Further, the Transwell invasion assay also showed that the motility of the CCA cell lines was significantly decreased in shNEAT1-transduced CCA cells compared to the control (Fig. 2D and E).

### Inhibition of NEAT1 Impaired CCA Cell Tumor Growth In Vivo

Next, we determined the effects of NEAT1 on in vivo tumor growth by xenografting shControl- or shNEAT1-transfected RBE and QBC939 cells into nude mice. We observed that both tumor volume (Fig. 3A and B) and tumor weight (Fig. 3C) were significantly decreased in mice xenografted with CCA cells with knocked down NEAT1. These data further supported the role of NEAT1 in CCA cell growth in vivo.

### NEAT1 Promotes Cholangiocarcinoma Metastasis Through Repression of E-Cadherin

Epithelial–mesenchymal transition (EMT) has been studied thoroughly in the past years. To illustrate the mechanism of metastasis for NEAT1, we checked the
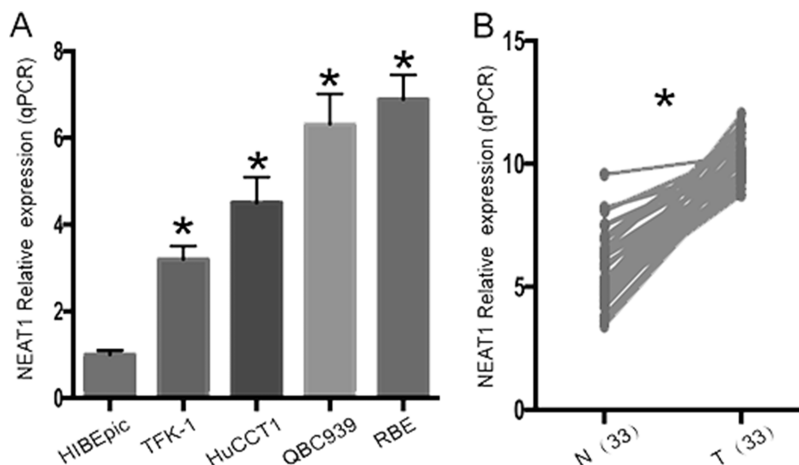


**Figure 1.** Enhanced nuclear paraspeckle assembly transcript 1 (NEAT1) expression in human cholangiocarcinoma (CCA). (A) Long noncoding RNA (lncRNA) NEAT1 abundance in nonmalignant cholangiocyte cell (HIBEpic) and four CCA cell lines (TFK1, HuCCT1, QBC939, and RBE). Data are represented as the mean±SD from three independent experiments. (B) Relative expression level of lncRNA NEAT1 in 33 paired benign and malignant cholangiocyte tissues. lncRNA NEAT1 expression was examined by quantitative real-time (qRT)-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. *$p < 0.05$, Student's $t$-test.
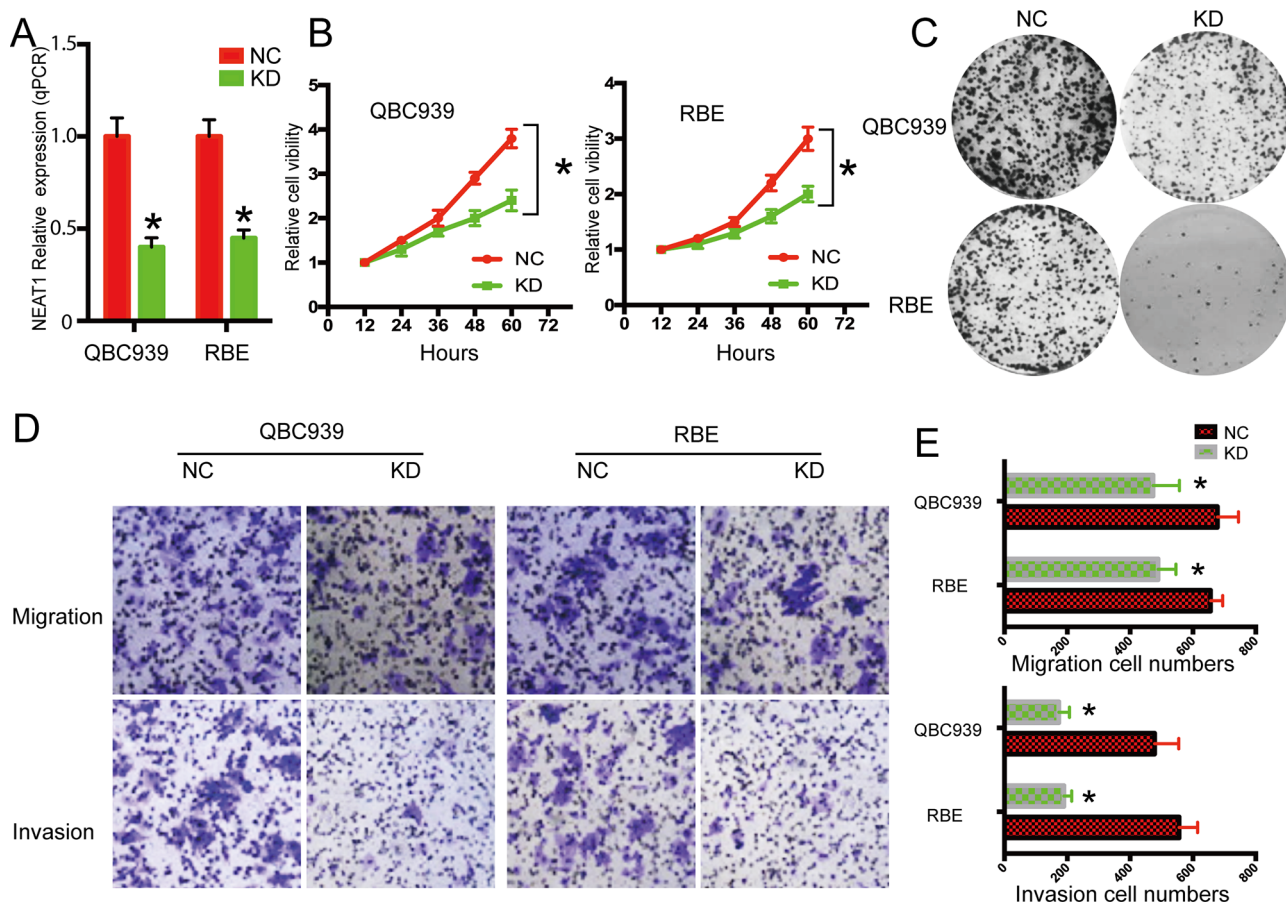
**Figure 2.** Knockdown of NEAT1 significantly inhibited cell proliferation, migration, and invasion in vitro. (A) NEAT1 expression in stable knockdown was confirmed by RT-PCR. (B) Cell counting kit-8 (CCK-8) assay was performed to measure proliferation of RBE and QBC939 cells. (C) Cell clonogenicity was measured by the colony formation. (D, E) Images of stained migratory and invasive QBC939 and RBE cells, and the average number of cells was calculated. *$p<0.05$, Student's $t$-test.

expression of several metastatic markers including EMT markers. We found that knockdown of NEAT1 significantly downregulated the levels of vimentin and N-cadherin but increased E-cadherin expression in the mRNA level in CCA (Fig. 4A). In addition, the protein level showed the same trend as the mRNA level (Fig. 4B). Previous studies determined that E-cadherin was associated with tumor progression and was an important predictive factor for cancer. These results indicated that NEAT1-mediated metastasis may be caused by repressing E-cadherin in CCA.

### NEAT1 Inhibits E-Cadherin Expression Partly Through Association With EZH2

To further confirm the association of NEAT1 and E-cadherin in CCA, we assessed E-cadherin expression in the 33 paired CCA tissues. NEAT1 expression was inversely correlated with E-cadherin expression ($p<0.001$) (Fig. 5A), but the mechanism is unknown. lncRNAs can regulate gene transcription and expression through histone

modifications, and EZH2 contains a potential lncRNA-binding site[16–18]. EZH2 is oncogenic in a wide variety of cancer types, functioning predominately as a transcriptional repressor that silences tumor suppressor gene targets[19]. It was reported that EZH2 suppresses E-cadherin expression[20]. There was a positive correlation of EZH2 and NEAT1 expression in CCA tissues, and EZH2 was also inversely correlated with E-cadherin expression (Fig. 5B and C). Therefore, we hypothesized that EZH2 might be recruited by NEAT1 to synergistically repress E-cadherin. To test this hypothesis, we first asked whether NEAT1 bound to EZH2 using a RIP assay. As shown in Figure 5D and E, there was a great enrichment for NEAT1 in the RBE and QBC939 cells using anti-EZH2, respectively, compared to the anti-IgG group. These results suggest that NEAT1 is physically associated with EZH2, and silencing EZH2 could increase E-cadherin transcription (Fig. 5F).

Next, we used ChIP to examine whether EZH2 could be recruited to the E-cadherin promoter by NEAT1.
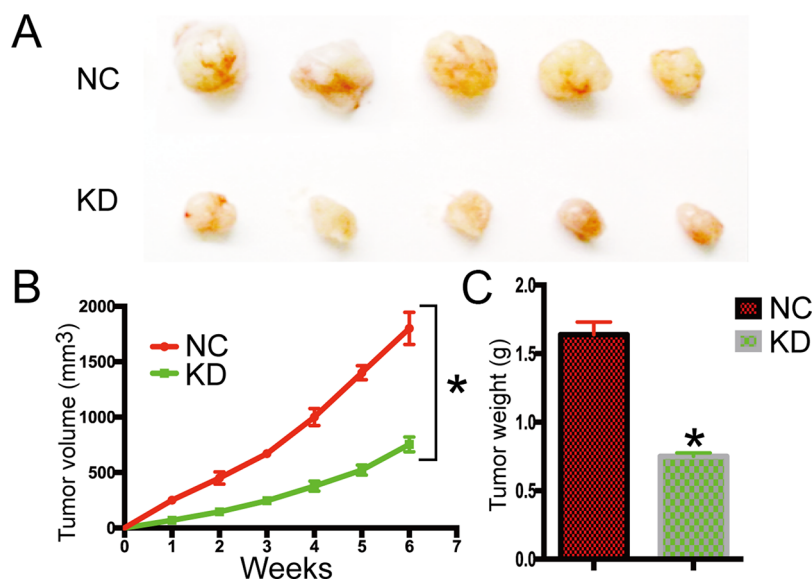
**Figure 3.** Knockdown of NEAT1 decreases proliferation of CCA in vivo. (A) Photographs of the xenograft tumor masses of RBE transduced with control or NEAT1 shRNA. (B) Tumor volumes were measured weekly from weeks 1 to 6 postinjection. (C) The average tumor weights harvested from nude mice infected with shControl lentiviral vector or shNEAT1 lentiviral vector are shown. *$p < 0.05$, Student's *t*-test.

We designed three primer pairs targeting the E-cadherin promoter (Fig. 5G) and assessed the enrichment of EZH2 after NEAT1 interference. Depletion of NEAT1 caused a reduction in EZH2 binding to the E-cadherin promoter (Fig. 5H and J). Momparler and colleagues found that EZH2 suppresses gene expression through H3K27me3[21]. We also performed ChIP using an H3K27me3 antibody and found decreased enrichment of H3K27me3 at the

E-cadherin promoter when compared to IgG antibody (Fig. 5I and J). Taken together, these data suggest that EZH2 is recruited by NEAT1 to the promoter of E-cadherin and that together they repress E-cadherin expression.

## DISCUSSION

Notwithstanding some diagnostic advances and therapeutic procedures that have been accomplished for CCA
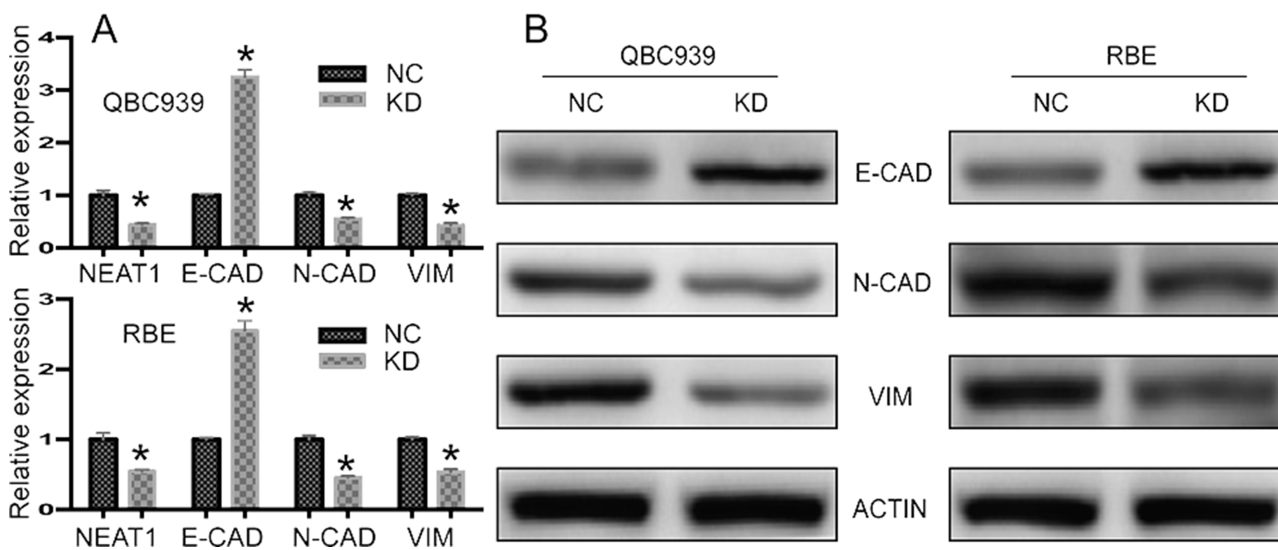


**Figure 4.** NEAT1 knockdown in CCA cells inhibits E-cadherin but increases N-cadherin and vimentin. (A) The mRNA expression of E-cadherin, N-cadherin, and vimentin after knocking down NEAT1. (B) The protein expression of E-cadherin, N-cadherin, and vimentin after knocking down NEAT1. *$p < 0.05$, Student's *t*-test.
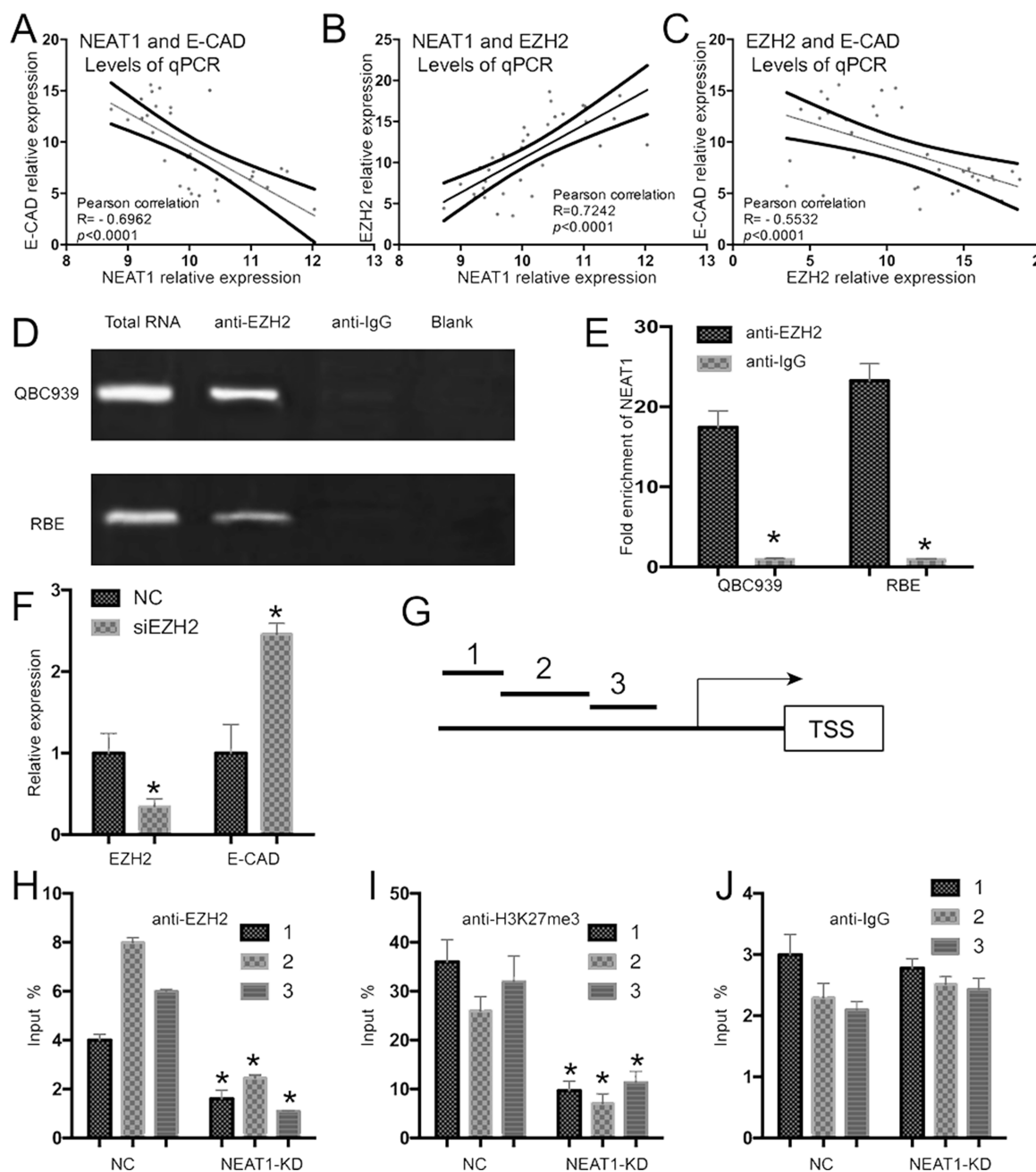
**Figure 5.** Enhancer of zeste homolog 2 (EZH2) is recruited by NEAT1 to bind to the E-cadherin promoter, suppressing E-cadherin expression. (A) Correlation of the expression of NEAT1 and E-cadherin in 33 CCA tissues. (B). Correlation of the expression of NEAT1 and EZH2 in 33 CCA tissues. (C) Correlation of the expression of EZH2 and E-cadherin in 33 CCA tissues. (D, E) RIP experiments were performed using the EZH2 antibody to immunoprecipitate RNA and a primer to detect NEAT1 RNA; compared to immunoglobulin G (IgG), the anti-EZH2 antibody group is rich in NEAT1. (F) EZH2 interference increased the E-cadherin mRNA level. (G) Chromatin immunoprecipitation (ChIP) assays were conducted on E-cadherin promoter region (primer sets 1–3) using the indicated antibodies. Enrichment is determined relative to input controls. (H–J) The targeted antibody EZH2 (H), H3K27me3 (I), and negative control IgG (J) were used for the ChIP assay. Inference of NEAT1 reduced the recruitment of EZH2 in the E-cadherin promoter region. $*p < 0.05$ compared with the control group.

in recent years, overall survival rates of CCA patients is quite poor due to recurrence and metastasis[22,23]. Hence, novel diagnostic and therapeutic approaches are necessary to improve the survival rate of CCA patients and to implement further insights into the pathogenesis of this deadly disease.

Recent advances in genome-wide analyses have exposed that more than 97% of the total human genome is transcribed into short or long noncoding RNAs with limited or no protein-coding capacity. Additionally, the roles of deregulated lncRNAs in human cancers have gained considerable attention in the past few years[24]. Many studies have revealed that lncRNAs are correlated with many functional aspects of cell biology, and there has been rising attention to their role in tumorigenesis. For example, lncRNA GAS5 has been shown to be significantly downregulated in hepatocellular carcinoma tissues[25]. lncRNA HOTAIR has been reported to be upregulated in breast cancer, pancreatic cancer, lung cancer, and gastric cancer, and high HOTAIR expression is associated with poor prognosis[26]. To the best of our knowledge, there has been limited study on the role of NEAT1 in CCA to date. Thus, further study about CCA-related lncRNAs in CCA is desirable for this deadly disease. Our study provides the first evidence that NEAT1 is significantly upregulated in CCA cell lines compared to normal HIBEpic cells. This further supported the finding that NEAT1 was upregulated in the CCA tumors compared to adjacent normal patient tissues. Additionally, we explored the biology of NEAT1 in CCA and showed that loss of NEAT1 expression modulating shRNA targeting NEAT1 could inhibit cell growth in vitro. The outstanding ability of NEAT1 to promote tumorigenesis supports that it plays an oncogenic role in CCA. Therefore, targeting NEAT1 may represent a promising therapeutic strategy for CCA treatment.

Low expression of E-cadherin in tumors is associated with tumor cell invasion and metastasis. Previous studies determined that downregulation of E-cadherin was associated with tumor progression and was an important predictive factor for cancer[27,28]. We found that silencing NEAT1 using shRNA increased E-cadherin expression. We also investigated and found that N-cadherin and vimentin expression was decreased after NEAT1 silencing in CCA cell lines. Moreover, NEAT1 expression correlated inversely with E-cadherin expression. We suggest that NEAT1 may promote progress of CCA by suppression of E-cadherin.

Weak expression of E-cadherin might be due to hypermethylation of the E-cadherin promoter[29]. The precise mechanisms by which lncRNAs function indicate that lncRNAs may play their role as signals, decoys, guides, or scaffolds for other regulatory proteins[30]. For example,

lincRNA-p21 suppressed the expression of p53-regulated genes by binding to the hnRNP-K complex[31]. lncRNA Gas5 binds to the DNA-binding domain of the glucocorticoid receptor by acting as a decoy[32]. HOTAIR joined PRC2 to the LSD1/CoREST/REST complex, thus reducing gene expression[33]. So which one is the mediator for NEAT1? As is known, EZH2 is oncogenic in a wide variation of cancer types, functioning predominately as a transcriptional repressor that silences tumor suppressor gene targets[34]. EZH2 could bind to the lncRNAs EBIC and HOTAIR[35,36], and epigenetic silencing of the lncRNA SPRY4 occurs in NSCLC cells through direct transcriptional repression mediated by EZH2[37]. It was discovered that EZH2 promoted tumor cell migration and invasion via epigenetic repression of E-cadherin in renal cell carcinoma. In our study, we found a positive correlation between EZH2 and NEAT1 expression in CCA tissues. NEAT1 was also inversely correlated with E-cadherin expression, and knockdown of EZH2 upregulated E-cadherin mRNA expression in CCA cell lines. We therefore hypothesized that NEAT1 regulated E-cadherin through an association with EZH2. Our RIP results indicated that EZH2 bound to NEAT1. Furthermore, our ChIP results showed that NEAT1 interference reduces EZH2 recruitment to the E-cadherin promoter. We hypothesized that EZH2 is recruited by NEAT1 and increases H3K27me3 at the E-cadherin promoter, thereby suppressing E-cadherin expression and leading to a malignant phenotype of enhanced migration and invasion.

In conclusion, we presented the first evidence that NEAT1 is an oncogene important for CCA. Our results exhibited that NEAT1 expression was elevated in CCA cell lines and tissues. We then explored the biology of NEAT1 in CCA and revealed that loss of NEAT1 expression modulating shRNA targeting NEAT1 could inhibit cell growth in vitro and in vivo. Ultimately, our data indicate that EZH2 recruitment by NEAT1 and subsequent involvement of the complex in the repression of E-cadherin promote CCA migration and invasion. Taken together, our results verify that NEAT1 may serve as a potential target for cancer therapeutics. Further studies are necessary to unravel the detailed mechanism and function of NEAT1 in CCA to pursue an lncRNA-targeted therapeutic strategy for this deadly disease.

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