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Original Article

Long noncoding RNA NEAT1 promotes the metastasis of osteosarcoma via interaction with the G9a-DNMT1-Snail complex

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Abstract: Osteosarcoma (OS) is the most common histological form of primary bone cancer. Long noncoding RNA nuclear enriched abundant transcript 1 (NEAT1) functions as an oncogene in some cancers. However, the functional role of NEAT1 in OS metastasis remains elusive. In the present study, we found that NEAT1 expression was significantly increased in OS tissues and cell lines. Overexpression of NEAT1 in OS tissues was correlated with higher clinical stage, distant metastasis and poorer prognosis. Loss- and gain-of-function assays showed that NEAT1 positively regulated metastasis *in vitro* and *in vivo*. In addition, ectopic expression of NEAT1 also induced epithelial-mesenchymal transition (EMT). Mechanistic studies revealed that NEAT1 epigenetically suppresses E-cadherin expression through association with G9a-DNMT1-Snail complex. Taken together, our study reveals a critical epigenetic mechanism underlying NEAT1-mediated metastasis.

Keywords: NEAT1, Snail, G9a, DNMT1, H3K9me2, DNA methylation

Introduction

Osteosarcoma (OS) is the most common histological form of primary bone cancer, which is prevalent in children and young adults [1]. Despite the significant improvement of OS treatment, the survival rate remains poor mainly due to lung metastasis [2, 3]. Similar to other cancers, OS metastasis is caused by the abnormal activation of different pro-metastasis genes and/or inactivation of anti-metastasis genes. For example, E-cadherin (encoded by *CDH1* gene) is a well-known classical member of the cadherin superfamily, which is a calcium-dependent cell-cell adhesion glycoprotein [4]. Decrease of E-cadherin expression is closely associated with the metastasis of many cancers, including hepatocellular carcinoma [5], breast cancer [6], colorectal cancer [7], thyroid cancer [8] and OS [9]. However, the underlying molecular mechanism of OS metastasis remains unclear. Understanding the molecular mechanisms will be helpful for providing new prevention of OS metastasis.

It has been revealed that more than 90% of the human genome DNA is thought to be transcribed, while only about 2% of it can encode proteins. Majority of these transcribed RNAs are termed as non-coding RNAs (ncRNAs) [10]. According to their transcript length, ncRNAs can be classified into 2 groups: small non-coding RNAs composed of less than 200 nucleotides and long non-coding RNAs (lncRNAs) consisting of more than 200 nucleotides. The small ncRNAs, such as microRNAs (miRNAs), function in the tumorigenesis, metastasis and chemoresistance of OS [11-14]. Recent studies demonstrated that lncRNAs are crucial regulators of epigenetic modulation, transcription and translation [15, 16]. LncRNA nuclear enriched abundant transcript 1 (NEAT1) functions as an oncogene in some cancers, which regulates cellular proliferation and metastasis [17, 18]. It has been reported that NEAT1 is associated with the growth and metastasis of prostate cancer, and is a downstream transcriptional target of estrogen receptor α [19]. Chromatin isolation by RNA purification (ChIRP) revealed that NEAT1 localizes to the promoter regions of genes involved in prostate cancer progression and increases chromatin marks of active transcription at these sites. However, the functional role

of NEAT1 in OS metastasis remains elusive. In the present study, we detected the expression level of NEAT1 in OS tissues and analyzed its association with clinicopathalogical features and prognosis. Subsequently, we investigated the biological role and molecular mechanisms of NEAT1 in OS metastasis.

Materials and methods

Cell culture

LM7, MG63, U2OS, 143b, KHOS and Nhost cells were purchased from the Cell Resource Center of the Institute of Basic Medical Sciences at the Chinese Academy of Medical Sciences. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin G and 100 U/ml streptomycin (Invitrogen).

Tissue samples

The OS tissues and corresponding noncancerous tissues were surgically retrieved from 76 OS patients from Cangzhou Central Hospital. All participating patients signed consent forms. All experiments involving human subjects were reviewed and approved by the Clinical Research & Ethics Committee at Cangzhou Central Hospital.

Construction of stable cells with overexpression or knockdown of NEAT1

The sequence of the effective shRNA was provided as follows: TCTCTAGGTTTGGCGCTAA. shRNAs against NEAT1 and control hairpins were cloned into pLKO.1 lentiviral vector. Cells were transfected with lentiviral constructs expressing above shRNAs for 24 hr. Cells were selected with puromycin for 1 week.

Full-length NEAT1 cDNA was cloned into pcDNA3.1 vector (pcDNA-NEAT1). Cells were transfected with empty vector or pcDNA-NEAT1. Stable clones were obtained by selection with G418.

siRNA transfection

Smartpool siRNA against human G9a and Snail were from Dharmacon. siRNA were transfected into cells by using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Specific qRT-PCR experiments were performed in the StepOne™ Real-Time PCR System using SYBR® Green Mixture (Takara) following the manufacturer's protocol. Data were normalized to ACTB or to control samples. Primers sequences for the detected genes were as follows: NEAT1-F: TTGAGCCTCCGGTCATACTA, NEAT1-R: GCTCCATCTACAAGGCATCAA; CDH1-F: CTCCCTTCACAGCAGAACTAAC, CDH1-R: CCACCTCTAAGGCCATCTTTG; ACTB-F: CACCAGGCACCCAGTTTAAT, ACTB-R: AGTCTCTGCTCTCTCTTCCTATG. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative quantification of gene expression levels.

RNA-immunoprecipitation (RIP)

RIP assay was performed using the EZ-magna RIP Immunoprecipitation Kit (Millipore), according to its manual. Snail (Abcam), G9a (Abcam), and DNMT1 (Abcam) antibodies were used for RIP.

RNA pull-down and mass spectrometry assay

RNA pull-down was performed as previously described [20]. *In vitro* biotin-labeled RNAs (NEAT1 and antisense NEAT1) were transcribed with the biotin RNA labeling mix (Roche) and T7 RNA polymerase (Roche) treated with RNase-free DNase I (Promega) and purified with RNeasy Mini Kit (QIAGEN). Biotinylated RNA was incubated with nuclear extracts of cells, and pull-down proteins were run on SDS-PAGE gels. Mass spectrometry followed.

Western blot

Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The PVDF membranes were incubated with E-cadherin (Cell Signaling), Snail (Abcam), G9a (Abcam), or DNMT1 (Abcam) antibodies and followed by incubation with secondary antibodies (Jackson). Signals were visualized with a chemiluminescence system.

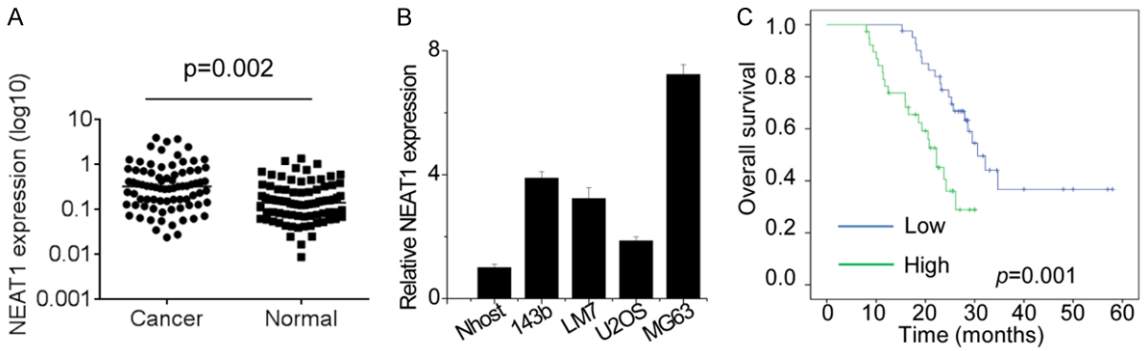


Figure 1. Increased NEAT1 expression predicts poor clinical outcome of patients with OS. A. The expression levels of NEAT1 in 76 pairs of OS and matched normal tissues were examined by qRT-PCR. B. The expression levels of NEAT1 in 6 different cell lines were detected by qRT-PCR. C. The Kaplan-Meier curves depicting OS patients with low and high expression for NEAT1. The median expression level of NEAT1 in OS tissues was used as the cutoff.

Table 1. The correlation between NEAT1 expression and clinicopathological variables in OS patients

Variables	NBAT1 expression levels		*P*
	High	Low	
Gender			
Male	16	15	0.808
Female	18	19	
Age			
>20	17	15	0.627
≤20	17	19	
Location			
Femur/Tibia	21	19	0.627
Elsewhere	13	15	
Tumor size (cm)			
≤5	19	20	0.806
<5	15	14	
Clinical stage			
I+IIA	10	22	0.004
IIB/III	24	12	
Distant Metastasis			
Yes	25	13	0.003
No	9	21	

P value was acquired by Pearson chi-square test. The median expression level was used as the cutoff.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously [20]. The primers for the *CDH1* promoter were: 5'-ACTCCAGGCTAGAGGGTCACC-3' and 5'-CCGCAAGCTCACAGGTGCTTTGCAGTTCC-3'.

Bisulfite sequencing PCR (BSP)

Genomic DNA (0.5 μg) was treated with sodium bisulfite using the EpiTect system (QIAGEN) according to the manual. Bisulfite-converted DNAs were used as templates for PCR amplification of the CpG islands in the *CDH1* promoter. All PCR products were purified from 1.5% agarose gels and cloned into the pMD-18T vector (Takara). Five randomly selected clones from each sample were selected for sequencing.

Migration and invasion assay

Transwell chambers (Corning Costar, USA) were used in the migration and invasion assays. For migration assay, 1×10^5 cells incubated in 200 μl serum-free medium were seeded in the upper chamber, while 500 μl DMEM supplemented with 10% FBS was added to the lower chamber. For the invasion assay, the upper chambers were pre-coated with Matrigel (BD Biosciences, USA) and 3×10^5 cells incubated in 200 μl serum-free medium were seeded in the upper chamber. The lower chamber was filled with full median. After 24 hours of incubation, cells in the upper chamber were removed, and the cells that had traversed the membrane were staining by crystal violet. All experiments were performed at least three times in triplicate.

Statistical analysis

The data were obtained from independent experiments that were repeated at least three

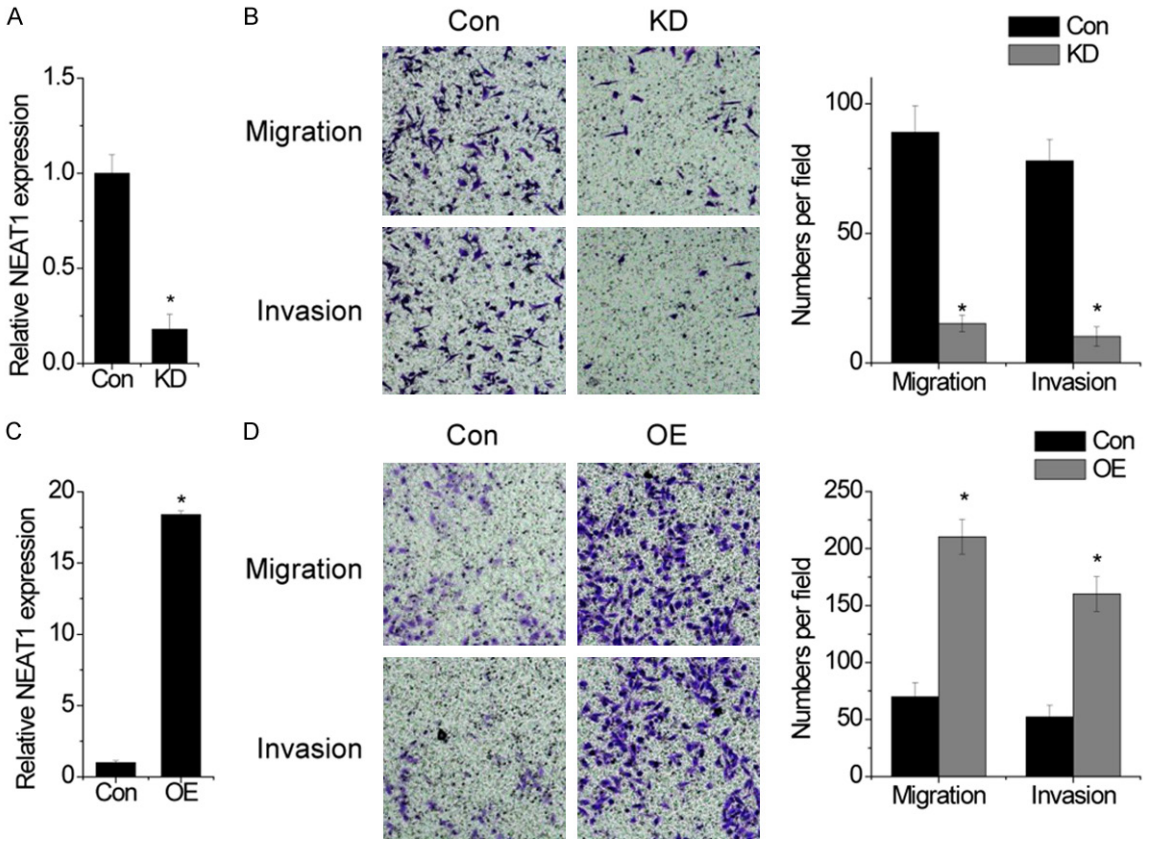


Figure 2. NEAT1 promotes OS cells migration and invasion *in vitro*. A. The MG63 stably knockdown (KD) NEAT1 were established. The relative expression of NEAT1 was detected by qRT-PCR. B. The effect of NEAT1 knockdown on MG63 cell migration and invasion. C. The U2OS stably overexpressing (OE) NEAT1 were established. The relative expression of NEAT1 was detected by qRT-PCR. D. The effect of NEAT1 overexpression on U2OS cell migration and invasion. Data are shown as mean ± std. **P*<0.05.

times, and each experimental group had three parallel groups. The differences between the experimental groups and the control groups were analyzed and determined using both a one-way ANOVA and Bonferroni's posttest ($P<0.05$). All statistical analyses and graphing were processed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Results

Increased NEAT1 expression predicts poor clinical outcome of patients with OS

At first, we performed qRT-PCR to determine the differential expression of NEAT1 in OS tissues and corresponding noncancerous tissues from 76 patients. The expression of NEAT1 was significantly increased in OS tissues compared with corresponding noncancerous tissues (**Figure 1A**). Moreover, we found that the NEAT1 expression was increased in OS cell lines (KHOS, 143b, LM7, U2OS, and MG63) compared with a normal osteoblast cell line Nhost (**Figure 1B**).

We next analyzed the clinical significance of NEAT1 expression in OS patients. As shown in **Table 1**, no significant differences were observed between NEAT1 expression and gender, age, location, or tumor size. Notably, NEAT1 expression in OS tissues was associated with clinical stage ($P=0.004$) and distant metastasis ($P=0.003$). The survival analysis also indicated that NEAT1 reduced the overall survival rate of patients with OS patients (**Figure 1C**). These results suggested that NEAT1 may play an oncogene in OS progression.

NEAT1 promotes OS cells migration and invasion in vitro

Based on above results, U2OS cells showing lowest endogenous NEAT1 expression and MG63 cells expressing highest endogenous NEAT1 level were used for the subsequent

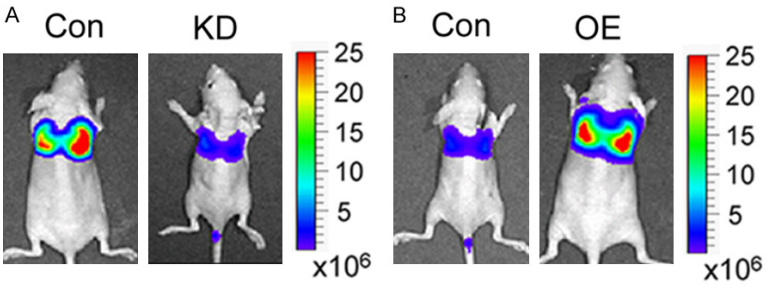


Figure 3. NEAT1 promotes the metastasis of OS cells *in vivo*. A. Representative images of luciferase signals of mice in each group 5 weeks after tail vein injection with 1×10^6 control and NEAT1 knockdown MG63 cells. B. Representative images of luciferase signals of mice in each group 5 weeks after tail vein injection with 1×10^6 control and NEAT1 overexpressing U2OS cells.

experiments. Migration and invasion are critical steps in tumor progression. We constructed stable MG63 cells with NEAT1 knockdown by shRNA-expressing lentiviruses. The qRT-PCR results indicated that this shRNA effectively knocked down NEAT1 expression (**Figure 2A**). Transwell assays and Matrigel-coated transwell assays demonstrated the positive effect of NEAT1 on OS cell migration and invasion, with inhibited cell migration and invasion after NEAT1 knockdown in MG63 cells (**Figure 2B**). In contrast, we construct stable U2OS cells with NEAT1 overexpression (**Figure 2C**) and found that overexpression of NEAT1 significantly enhanced the migratory and invasive ability (**Figure 2D**). Together, NEAT1 facilitates OS cells migration and invasion in vitro.

NEAT1 promotes the metastasis of OS cells in vivo

We next investigated the effects of NEAT1 on OS metastatic ability *in vivo*. To establish a metastatic cancer model *in vivo*, above OS cells were labeled with firefly luciferase and injected into tail vein of nude mice. A bioluminescent signal detection was performed at 10 weeks after tail vein injection. We found that cells with NEAT1 overexpression showed stronger signals of pulmonary metastasis than control cells (**Figure 3A**), whereas NEAT1 knockdown exhibited the opposite effect (**Figure 3B**). Taken together, these data demonstrated that NEAT1 promotes OS metastasis *in vivo*.

NEAT1 induces epithelial-mesenchymal transition in OS cells

Epithelial-mesenchymal transition (EMT) plays a critical role in metastasis. Specifically, EMT induces tumor-associated epithelial cells to obtain mesenchymal features, which results in reduced cell-cell contact and increased motility. Up-regulation of NEAT1 in U2OS cells resulted in the decreased expression of epithelial markers (E-cadherin and α-catenin) and increased expression of mesenchymal markers (Vimentin and N-cadherin), as evidenced by immunofluorescence (IF), western blotting analysis, and real-time PCR (**Figure 4A-C**). These findings suggested that NEAT1 induced EMT in OS cells.

NEAT1 interacts with G9a-DNMT1-Snail complex

LncRNAs are considered to exert their functions through RNA-interacting proteins that regulate gene expression by various mechanisms. Therefore, we performed an RNA pull-down assay with biotin-labeled NEAT1 to search for potential NEAT1-associated proteins. G9a, DNMT1 and Snail were identified to potentially interact with NEAT1 in OS cells. G9a, DNMT1 and Snail can form a transcrptional repressive complex to induce EMT [21]. The interaction of NEAT1 with G9a-DNMT1-Snail complex was further validated by RNA immunoprecipitation (RIP) (**Figure 5A**). Furthermore, we performed RNA pull-down assay to confirm the association between NEAT1 and G9a-DNMT1-Snail complex (**Figure 5B**). Consistent with the result of RIP assay, G9a, DNMT1 and Snail could be pulled down by biotinylated NEAT1 RNA.

NEAT1 suppresses E-cadherin expression through association with G9a-DNMT1-Snail complex

Next, we determined the functional relationship between NEAT1 and the G9a-DNMT1-Snail complex. NEAT1 overexpression did not influence protein level of G9a-DNMT1-Snail (data not shown), suggesting that NEAT1 was not involved in the post-translational regulation of the G9a-DNMT1-Snail complex. We hypothesized that NEAT1 may have an effect on transcriptional suppression mediated by the G9a-DNMT1-Snail complex. *CDH1* (encode E-cadherin protein) is well-known target gene of the

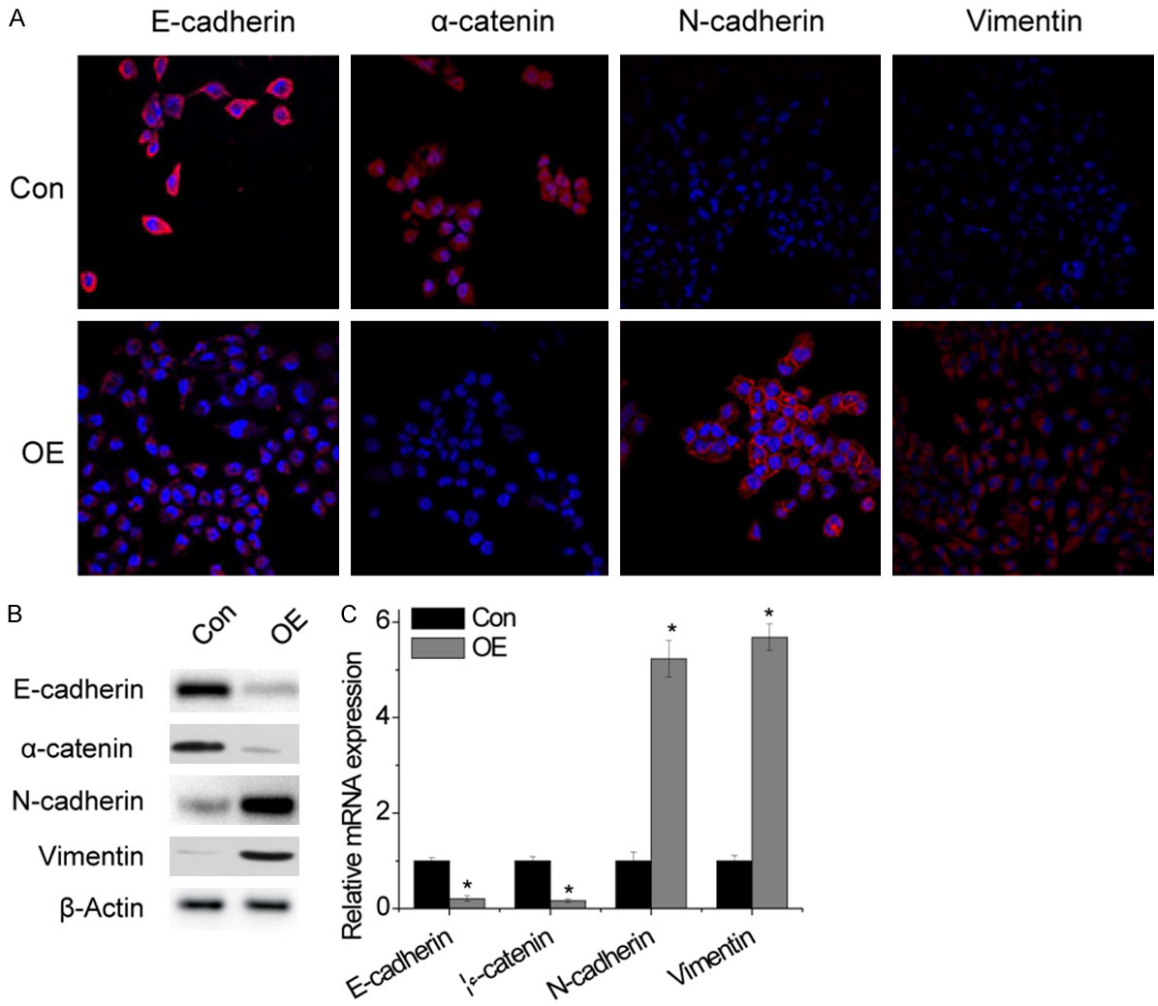


Figure 4. NEAT1 induces epithelial-mesenchymal transition in OS cells. A. Immunofluorescence was used to compare the expression levels of epithelial and mesenchymal markers between control and NEAT1-overepxressing cells. B. Expression of epithelial markers and mesenchymal markers were compared by western blot analysis between control and NEAT1-overepxressing cells. β-Actin was used as a loading control. C. Expression of epithelial markers and mesenchymal markers were compared by qRT-PCR between control and NEAT1-overepxressing cells. Data are shown as mean ± std. **P*<0.05.

G9a-DNMT1-Snail complex and the hall marker of EMT. As shown in **Figure 4**, NEAT1 overexpression dramatically inhibits E-cadherin expression. To further confirm the regulatory relationship between NEAT1 and E-cadherin expression, we cloned the *CDH1* promoter to reporter vector and performed dual-luciferase reporter assays to detect whether NEAT1 have an effect on *CDH1* promoter activity. Consistently, NEAT1 silencing increased *CDH1* promoter luciferase activity (**Figure 6A**), while NEAT1 overexpression suppressed its activity (**Figure 6B**). We also determined whether NEAT1 have an effect on the Snail-binding level, G9a-induced H3K9me2 level and DNMT1-mediated DNA methylation in the *CDH1* promoter by using ChIP and BSP assay, respectively. We found that dowregulation of NEAT1 suppressed the binding level of G9a, Snail and DNMT1 to the *CDH1* promoter, and decreased the H3K9me2 and DNA methylation levels across the promoter regions of *CDH1* (**Figure 6C** and **6D**). Furthermore, knockdown of Snail or G9a or DNMT1 partially rescued the *CDH1* mRNA expression and the luciferase activity of *CDH1* promoter decreased by NEAT1 upregulation (**Figure 6E** and **6F**). Consistent with these results, knockdown of Snail or G9a significantly suppressed NEAT1-induced H3K9me2 on the *CDH1* promoter (**Figure 6G**). Dowregulation of

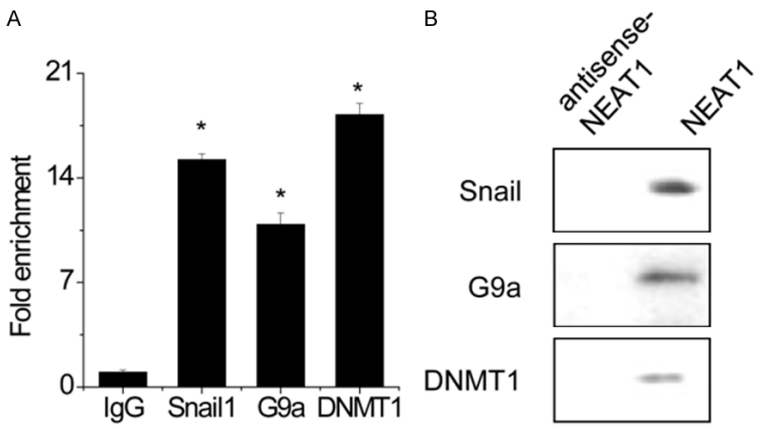


Figure 5. NEAT1 interacts with G9a-DNMT1-Snail complex. A. The interaction between NEAT1 and G9a-DNMT1-Snail complex was confirmed by RIP assay. B. Western blot of G9a, DNMT1 and Snail expression in protein complexes pulled down by NEAT1 or antisense control from cell extracts. Data are shown as mean ± std. **P*<0.05.

Snail or G9a or DNMT1 markedly suppressed the NEAT1-mediated DNA methylation on the *CDH1* promoter (**Figure 6H**). These data suggest that *CDH1* transcription reduced by NEAT1 is closely associated with Snail-G9a-DNMTs complex.

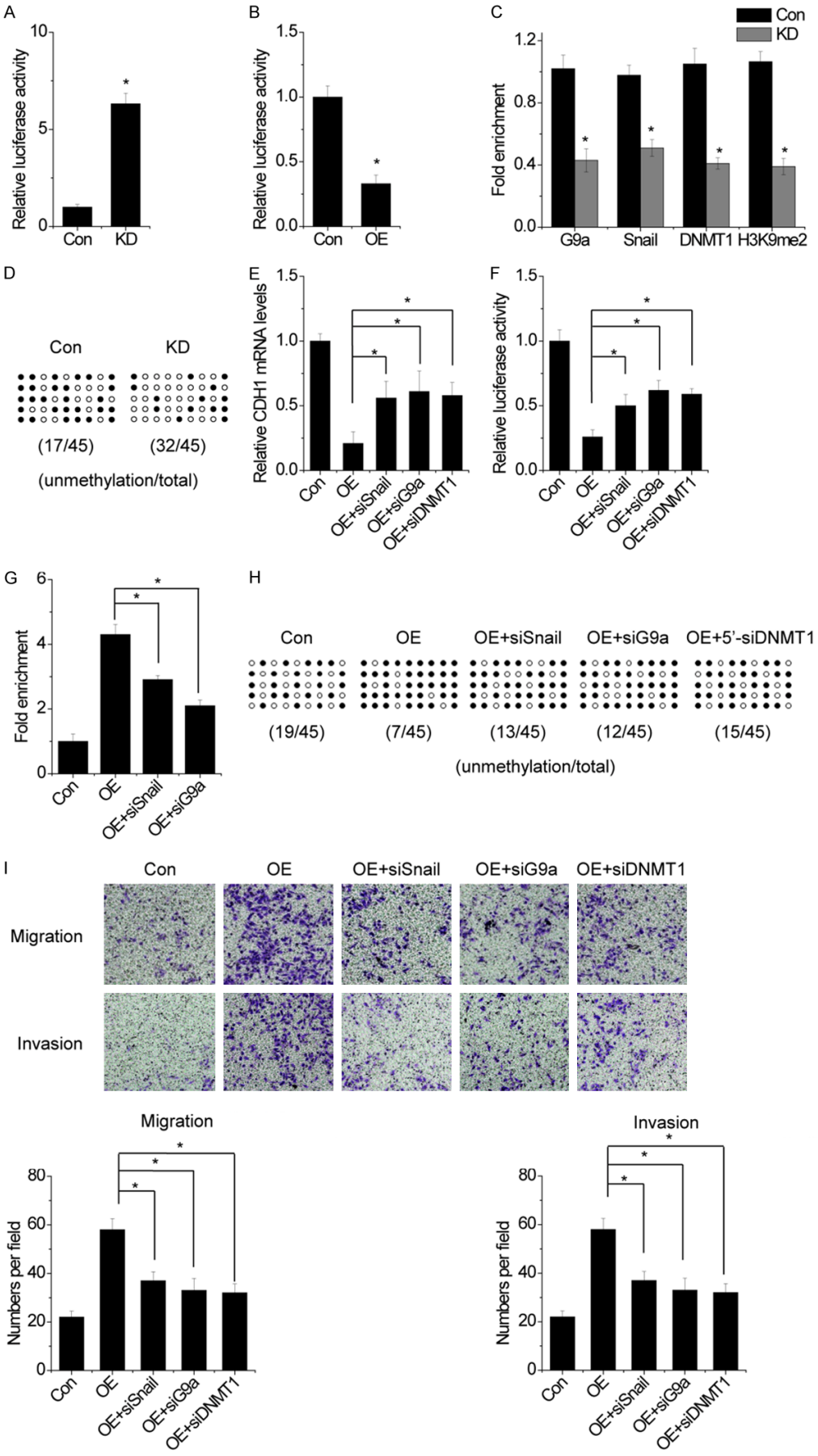
Finally, we tested whether NEAT1 promotes cell migration and invasion via association with the Snail-G9a-DNMTs complex. Our results showed that depletion of Snail-G9a-DNMT1 complex markedly suppressed the NEAT1-induced migration and invasion (**Figure 6I**). These results strongly support our finding that Snail-G9a-DNMTs complex is essential for the functional role of NEAT1 in OS progression.

Discussion

Increasing evidences reported that many lncRNAs are frequently dysregulated in cancers. These differential expression lncRNAs are closely related to tumorigenesis, tumor metastasis and poor prognosis, serving as roles of oncogenes or tumor suppressor genes [22]. Thus, clarifying the biological and molecular mechanisms of lncRNAs in cancer will be helpful for cancer treatment.

In this study, we analyzed the expression pattern of a novel lncRNA, NEAT1, in OS tissues and their corresponding nontumorous tissues. The results demonstrated that NEAT1 was upregulated in OS tissues and was closely correlated with clinical stage, distant metastasis and poor overall survival of patients with OS. In addition, we revealed the functional role of NEAT1 in OS cells by applying loss- and gain-of-function approaches. NEAT1 knockdown could suppress cell migration and invasion in OS cells *in vitro*, as well as inhibit tumor metastasis *in vivo*, whereas NEAT1 overexpression showed the opposite effect. This is the first report to demonstrate the functional significance of NEAT1 expression in OS, and our findings indicate that NEAT1 functions as an oncogene and promotes OS malignant progression. Therefore, NEAT1 may be a promising diagnostic and prognostic marker and therapeutic target for OS.

EMT is a phenotypic conversion linked with metastasis. This phenotypic and cellular plasticity of EMT is determined by epigenetic mechanisms through DNA methylation and histone modifications, which causes suppression of E-cadherin, the hallmark of EMT [23-25]. Many transcription factors, such as Twist, ZEB1 and Snail, are also involved in the E-cadherin suppression and the induction of EMT [26]. A recent study have reported that Snail interacted with G9a, a major chromatin methyltransferase responsible for histone H3 on lysine 9 (H3K9me2), and recruited G9a and DNA methyltransferases (DNMTs) to the *CDH1* promoter for transcriptional suppression [21]. LncRNAs are considered to exert their functions through RNA-interacting proteins that regulate gene expression by epigenetic mechanism. In our present study, we found that NEAT1 enhanced cell migration and invasion, and induced EMT. Mechanistically, our results demonstrated that NEAT1 associated with the G9a-DNMT1-Snail complex to suppress *CDH1* transcription. To uncover the epigenetic mechanism of *CDH1* silence, we examined the histone modification and DNA methylation status of the *CDH1* promoter. NEAT1 increased the H3K9me2 and DNA methylation level of the *CDH1* promoter. Furthermore, NEAT1 knockdown significantly decreased the occupancy of G9a-DNMT1-Snail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Relative luciferase activity
Con
KD
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D
Con
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(17/45)
(32/45)
(unmethylation/total)
E
Relative CDH1 mRNA levels
Con
OE
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OE+siG9a
OE+siDNMT1
F
Relative luciferase activity
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OE
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Figure 6. NEAT1 suppresses E-cadherin expression through association with G9a-DNMT1-Snail complex. A. The luciferase activity of *CDH1* promoter in control and NEAT1-silencing cells. B. The luciferase activity of *CDH1* promoter in control and NEAT1-overexpressing cells. C. ChIP assay for *CDH1* promoter by using G9a, Snail, DNMT1, or H3K9me2 antibodies in control or NEAT1-silencing cells. D. Schematic diagram showing CpG dinucleotides at the promoter region of *CDH1* in control or NEAT1 knockdown cells. Methylation of the *CDH1* promoter was analyzed by bisulfite sequencing. Hollow dot was defined as unmethylation, else as methylation. E. The relative expression of *CDH1* mRNA in control or NEAT1-overexpressing cells with or without siRNA against Snail (siSnail) or G9a (siG9a) or DNMT1 (siDNMT1). F. The luciferase activity of *CDH1* promoter in control or NEAT1-overexpressing cells with or without siRNA against Snail (siSnail) or G9a (siG9a) or DNMT1 (siDNMT1). G. H3K9me2 level across *CDH1* promoter in control or NEAT1-overexpressing cells with or without siRNA against Snail (siSnail) or G9a (siG9a) was detected by ChIP assay. H. Schematic diagram showing CpG dinucleotides at the promoter region of *CDH1* in control or NEAT1-overexpressing cells with or without siRNA against Snail (siSnail) or G9a (siG9a) or DNMT1 (siDNMT1). Methylation of the *CDH1* promoter was analyzed by bisulfite sequencing. Hollow dot was defined as unmethylation, else as methylation. I. The number of migrated and invaded NEAT1-overexpressing cells with or without siRNA against Snail (siSnail) or G9a (siG9a) or DNMT1 (siDNMT1). Data are shown as mean ± std. **P*<0.05.

complex on the promoter regions of *CDH1*, suggesting that NEAT1 was essential for G9a-DNMT1-Snail-induced *CDH1* suppression. Our present study provides novel insights into the epigenetic program involving lncRNA in suppression of *CDH1* transcription. A recent study demonstrated that NEAT1 induced EMT through miR-204/ZEB1 axis in nasopharyngeal carcinoma [27]. This study implies that NEAT1 may regulate E-cadherin expression through diverse mechanisms in different cancers.

In summary, our study showed that lncRNA NEAT1 expression is upregulated in OS tissues, is associated with poor prognosis of patients with HCC. Its effects on OS cell metastasis *in vitro* and *in vivo* indicate that it exhibits oncogene properties in OS progression. NEAT1 exerts its function through inducing EMT via association with G9a-DNMT1-Snail complex. NEAT1 and Snail-G9a-DNMTs complex may be a promising therapeutic approach that target metastatic OS.

Disclosure of conflict of interest

None.

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