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Original Articles

Long non-coding RNA NEAT1 promotes colorectal cancer progression by competitively binding miR-34a with SIRT1 and enhancing the Wnt/β-catenin signaling pathway

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ABSTRACT

In recent years, accumulating evidence has indicated that long non-coding RNAs (lncRNAs) are powerful factors influencing the progression of multiple malignancies. Although a relationship between the lncRNA NEAT1 (nuclear enriched abundant transcript 1) and colorectal cancer has previously been reported, the functional mechanism underlying the involvement of NEAT1 in colorectal cancer remains unknown. In this study, we report that NEAT1 expression is up-regulated in colorectal cancer tissues, which correlates with advanced clinical features, poor overall survival and disease free survival. Up-regulated NEAT1 promotes cell proliferation and metastasis of colorectal cancer both *in vitro and in vivo*. Moreover, NEAT1 functions as an oncogene influencing cell viability and invasion in part by serving as a competing endogenous RNA (ceRNAs) modulating miRNA-34a expression, leading to subsequent repression of the miR-34a/SIRT1 axis and activation of the Wnt/β-catenin signaling pathway. Taken together, our study demonstrates that the lncRNA NEAT1 may serve as a prognostic biomarker and a potential therapeutic target in colorectal cancer.

1. Introduction

Colorectal cancer (CRC) is a common cause of global cancer-related death due to late tumor identification and rapid progression [1,2]. Despite significant improvements in current clinical treatments for CRC, such as adjuvant chemo-radiotherapies and immunological therapy, the prognosis of patients with advanced-stage disease remains poor, and the 5-year survival rate is far from satisfactory [1,3]. Therefore, greater understanding of the mechanisms of CRC initiation and progression is urgently needed to improve the survival rate of affected patients.

Long non-coding RNAs (lncRNAs), a class of non-coding RNAs with more than 200 nucleotides in length, are reportedly involved in a large range of biological processes, including cell differentiation, proliferation, and tumorigenesis [4,5]. Increasing evidence suggests that lncRNAs, similar to protein-coding genes, may mediate oncogenic or tumor-suppressing effects and function as a new class of colorectal cancer biomarkers and therapeutic targets [6,7]. LncRNA NEAT1 (nuclear enriched abundant transcript 1) is a novel oncogenic gene in diverse types of tumorigenesis, including breast cancer, gastric cancer and non-small cell lung cancer [8–10]. However, little is known concerning whether NEAT1 affects biological behavior and the underlying mechanisms of colorectal cancer.

In the present study, our data showed that lncRNA NEAT1 overexpression is a characteristic molecular change and a reliable prognostic factor for the survival of CRC patients. We further explored the effects of NEAT1 on the phenotypes of CRC cells both *in vitro and in vivo*. Moreover, underlying mechanistic analysis revealed that NEAT1 could act as ceRNAs to regulate SIRT1 expression by sponging miR-34a in CRC. Taken together, our study provides the first evidence of the existence of a NEAT1-miR-34a/SIRT1-Wnt/β-catenin signaling axis, which can be used as a potential therapeutic target for colorectal cancer.

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2. Materials and methods

2.1. In silico analysis using the gene expression omnibus (GEO)

To determine the expression pattern of NEAT1 in colorectal cancer, two datasets (GSE20916 and GSE9348) in GEO (http://www.ncbi.nlm.nih.gov/gds/) were used. Data extraction was performed with R 3.0.2 software. We compared NEAT1 gene expression in colorectal cancer tissues with normal tissues according to the standard procedures as previously described [11].

2.2. Clinical samples

A total of 100 pairs of CRC tumor and matched non-tumor tissues were collected at the Department of Gastrointestinal Surgery, Ren Ji Hospital between January 2012 and December 2016. None of the patients had received tumor-specific therapy prior to diagnosis. The cases of CRC were selected for further study only if clinical data were available, and the patients were verified as having CRC via H&E staining after surgery. The follow-up time was calculated from the date of surgery to the date of death, or the last known follow-up timepoint. All patients were well informed, the process was approved by Ethics Committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine, and written informed consent was obtained from each patient.

2.3. Cell culture and transfection

Human CRC cell lines SW620, SW480, HCT116, HT29, CaCo-2, LoVo and Colo205 were purchased from American Type Culture Collection (ATCC) and maintained in our laboratory. All of these cells were cultured in specific medium supplemented with 10% (volume/volume) fetal bovine serum (FBS) and 1% antibiotics at 37 °C in a humidified incubator under 5% CO_2 condition.

Transfections were performed using lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's instructions. MiR-34a mimics, miR-34a inhibitors and miRNA controls were obtained from GenePharma Technology (Shanghai, China). Small interfering RNAs (siRNAs) targeting NEAT1, SIRT1 and a negative control were also obtained from GenePharma Technology (Shanghai, China). NEAT1 overexpression plasmids (pcDNA3.1-NEAT1) were purchased from Genearray Biotechnology (Shanghai, China). The transfections were performed according to the manufacturer's protocol. HT29 cells and SW480 cells stably expressing NEAT1 were obtained using G418 selection. Briefly, the HT29 and SW480 cells (1×10^5 cells/cm^2 in 24-well plates) were then grown in complete medium containing 250 mg/ml G418 (Sigma-Aldrich, USA). Following transient transfection for 48 h, cells were passaged 1:10 (v/v) and cultured in medium supplemented with G418 at 600 μg/ml for 4 weeks. The surviving clones were selected and maintained in medium containing G418 at a concentration of 300 μg/ml. The subcloned cells expressing NEAT1 were termed HT29-NEAT1-cDNA cells or SW480-NEAT1-cDNA cells, respectively. NEAT1 cloning was confirmed using RT-qPCR.

2.4. Total RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from tissues and cells using Trizol reagent (Invitrogen, USA). cDNA was synthesized using a microRNA Reverse Transcription Kit (Promega, USA) or a PrimeScript RT-PCR kit (Takara, Japan). RT-qPCR was performed using a StepOne Real-Time PCR System (Applied Biosystems, USA). The primers used in this study are shown in Table 1. Relative expression levels were determined by normalizing the expression of each Ct value to U6 Ct or GAPDH Ct values, and data were analyzed according to the $2^{-\Delta\Delta}$Ct method.

Table 1
Quantitative Real-time PCR primers used in this study.

Gene name	Primer sequence (5′ to3′)	Amplicon size
NEAT1	Forward: GGCAGGTCTAGTTTGGGCAT Reverse: CCTCATCCCTCCCAGTACCA	278 bp
SIRT1	Forward: TGCTGGCCTAATAGAGTGGCA Reverse: CTCAGCGCCATGGAAAATGT	102 bp
MYCN	Forward: ACAGTCATCTGTCTGGACGC Reverse: TCGGAAGCAGAAACAGTCCC	319bp
JAG1	Forward: ACGGGAAGTGCAAGAGTCAG Reverse: GTTTCACAGTAGGCCCCCTC	193bp
ZDHHC17	Forward: GAGTACGATACCGAAGCGGG Reverse: ATTGATGGCAGCCCAATGGA	252bp
ACSL1	Forward: TGACTTGTGCCCTGAGAGAC Reverse: GTTGGTCGGAAGAGTACGCA	257bp
GAPDH	Forward: TGAAGGTCGGAGTCAACGGA Reverse: CCTGGAAGATGGTGATGGGAT	225 bp

2.5. Cell proliferation assay

A density of 3000–4000 indicated CRC cells/well upon different treatments were seeded in a 96-well cell culture plate and grown at 37 °C overnight. The cell viability was quantified via Cell Counting Kit-8 assay (CCK-8, Japan). Briefly, on the day of measuring the growth rate of treated cells, 100 μl of spent medium was replaced with an equal volume of fresh medium containing 10% CCK-8, cells were incubated at 37 °C for 1 h, and the absorbance was subsequently detected at 450 nm with a microplate reader.

2.6. Transwell assay

Briefly, 4–5 × 10^4 cells suspended in serum-free medium were plated on the top of each chamber, while medium containing 20% FBS was placed in the lower chamber. After incubation for 48 h, the chambers were disassembled, the noninvading cells that remained in the upper chamber were removed, and the membranes were stained with a 2% crystal violet solution for 30 min. Then, cells that had migrated across the membrane were counted in five random visual fields using a light microscope.

2.7. Western blot

All proteins were resolved via 10% SDS-PAGE and were then transferred onto a PVDF membrane. Membranes were incubated with blocking buffer for 90 min at room temperature and then incubated with an antibody targeting SIRT1 (1:1000, ab110304, Abcam, UK), β-catenin (1:1000, ab32572, Abcam, UK), c-myc (1:1000, ab32072, Abcam, UK), cyclin D1 (1:1000, ab134175, Abcam, UK), E-cadherin (1:1000, ab1416, Abcam, UK) or GAPDH (1:1000, ab8245, Abcam, UK) overnight at 4 °C. The membranes were washed and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. Protein expression was assessed by enhanced chemiluminescence and exposed to chemiluminescent film. LabWorks image acquisition and analysis software was used to quantify band intensities.

2.8. Luciferase reporter assays

Putative wild-type (wt) and mutant (mut) miR-34a-binding sites in the 3'-UTR of NEAT1 or SIRT1 mRNA, termed pmirGLO-NEAT1-wt or pmirGLO-NEAT1-mut, and pmirGLO-SIRT1-wt or pmirGLO-SIRT1-mut, were cloned into a pmirGLO-Report luciferase vector (Genearray Biotechnology, China). The reporter plasmid was transiently transfected into HCT116 and SW620 cells in the presence of either miR-34a mimics and/or pcDNA3.1-NEAT1. After 48 h incubation, luciferase activity was assessed using a dual-luciferase reporter-assay system (Promega, USA).

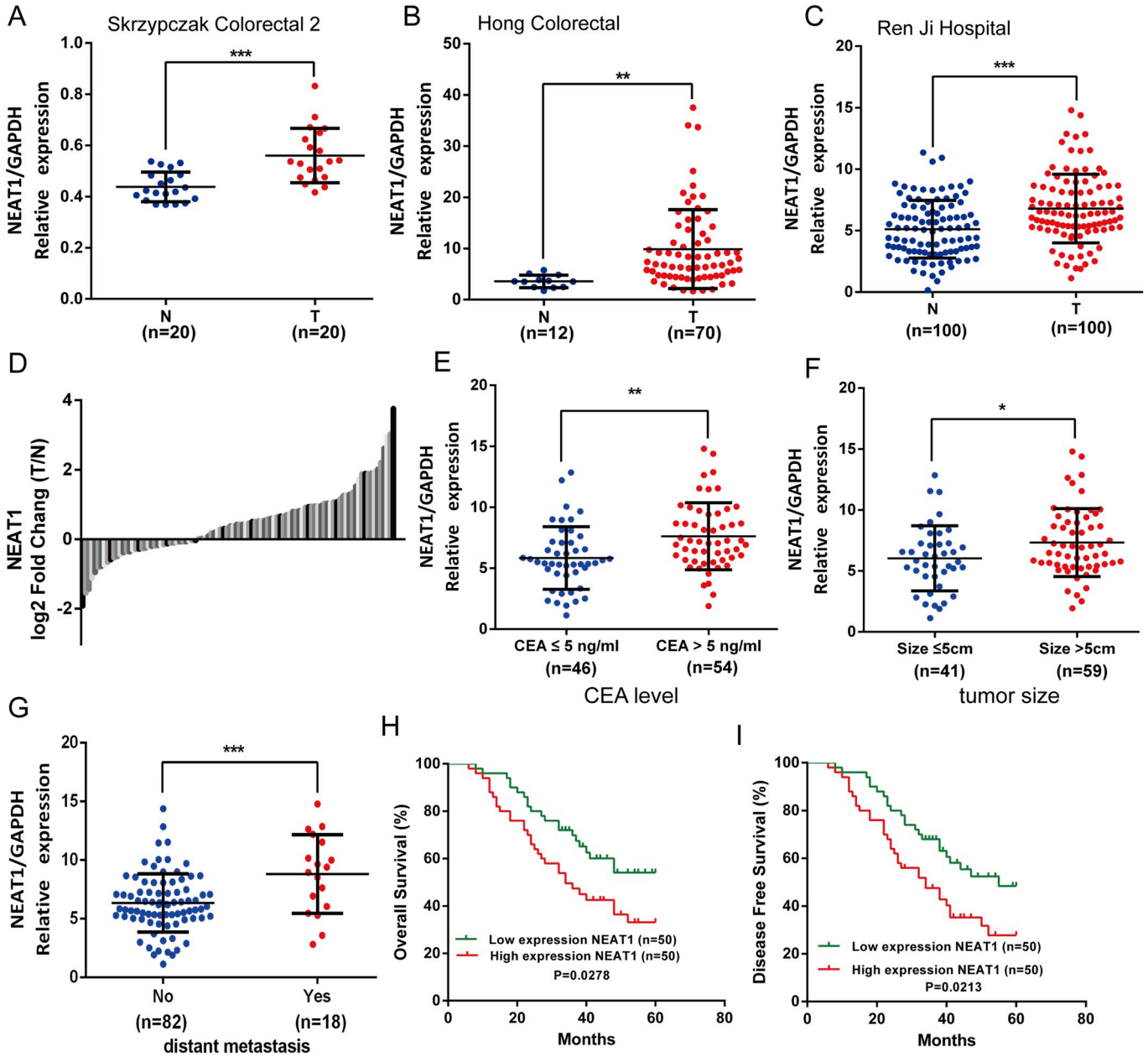

Fig. 1. NEAT1 is up-regulated in CRC and correlated with prognosis of CRC patients. (A–B): Detection of NEAT1 expression in dataset GSE20916, GSE9348. **(C–D):** The transcription level of NEAT1 in 100 matched CRC tissues (T) and adjacent normal tissues (N) by RT-qPCR and normalized by an endogenous control, GAPDH RNA. **(E–G):** The NEAT1 expression level was closely correlated with the CEA) level, tumor size and distant metastasis. **(H–I):** Kaplan-Meier analyses indicated that overall survival (OS) and disease-free survival (DFS) in the patients with high NEAT1 expression level was significantly shorter than that of the low expression group. *P < 0.05, **P < 0.01, ***P < 0.001.

2.9. Tumorigenesis in nude mice

BALB/c nude mice (4–6 weeks) were housed and maintained under specific pathogen-free conditions and used in accordance with institutional guidelines approved by the Use Committee for Animal Care. For xenografts, 5×10^6 HT29-NEAT1-cDNA cells (transfected with miR-34a agomir or control agomir) were subcutaneously injected into nude mice (n = 6 for each group). Mice were euthanized 25 days after the initial injection, and tumors were excised. Xenotransplanted tumors were measured via calipers every 5 days for 25 days, and mean values were calculated and plotted with analysis of variance.

2.10. Statistical analyses

Student's t testing or chi-squared testing was used to analyze the data. Survival curves were constructed using the Kaplan-Meier method and analyzed by the log-rank test. The results are presented as the means ± SD unless otherwise indicated. Probability values < 0.05 were considered statistically significant.

3. Results

3.1. LncRNA NEAT1 expression levels in CRC tissues

To determine NEAT1 expression levels and their prognostic value in CRC patients, we first analyzed two independent microarray datasets

Table 2
Correlations between NEAT1 expression and clinicopathologic features in 100 colorectal cancer patients.

Clinicopathological feature	Total 100	Expression of NEAT1		P value (χ^2 test)
		Low (n = 50, 50%)	High (n = 50, 50%)	
Age(years)				
< 65	50	24 (48.00)	26 (52.00)	0.689
≥65	50	26 (52.00)	24 (48.00)	
Sex				
Male	53	28 (52.83)	25 (47.17)	0.548
Female	47	22 (46.81)	25 (53.19)	
Tumor location				
Rectum	51	24 (47.06)	27 (52.94)	0.548
Colon	49	26 (53.06)	23 (46.94)	
CEA level				
≤5 ng/ml	46	30 (65.22)	16 (34.78)	**0.005**
> 5 ng/ml	54	20 (37.04)	34 (62.96)	
Lymph node metastasis				
Absent	41	23 (43.90)	18 (56.10)	0.309
Present	59	27 (54.24)	32 (45.76)	
Tumor size				
≤5 cm	60	35 (58.33)	25 (41.67)	**0.041**
> 5 cm	40	15 (37.50)	25 (62.50)	
Distant metastasis				
Absent	82	45 (54.88)	37 (45.12)	**0.037**
Present	18	5 (27.78)	13 (72.22)	
TNM stage				
Stage I-II	54	32 (59.26)	22(40.74)	**0.045**
Stage III-IV	46	18 (39.13)	28 (60.87)	

Values in parentheses indicate percentage values. The bold number represents the P-values with significant differences.

from GEO database [12,13]. The results demonstrated that NEAT1 expression level were significantly increased in tumor tissues compared with normal tissues in both independent datasets (P < 0.000, P = 0.006), (Fig. 1A and B). Then we examined expression of NEAT1 in 100 matched CRC tumor and non-tumor tissues. Consistent with the data from GEO database, NEAT1 levels were significantly higher in CRC tissues than in adjacent normal tissues (p < 0.001) (Fig. 1C and D). In addition, the NEAT1 expression level was closely correlated with the carcinoembryonic antigen (CEA) level (p = 0.001), tumor size (p = 0.022) and distant metastasis (p < 0.001) (Fig. 1E–G). Next, we divided the 100 cases of CRC tumors into a NEAT1 high expression group (n = 50) and a NEAT1 low expression group (n = 50), according to the median expression level of NEAT1. Kaplan-Meier survival curve analysis and the log-rank test showed that the overall survival (OS) and disease-free survival (DFS) rate of CRC patients with low NEAT1 expression was higher than that of patients with high NEAT1 expression (p = 0.0278 and p = 0.0213) (Fig. 1H and I). These results indicated that NEAT1 expression levels were significantly increased in colorectal cancer and associated with a poorer prognosis.

3.2. Relationship between lncRNA NEAT1 expression and clinicopathological features of CRC

To determine the clinical significance of NEAT1 expression, we next analyzed the association of NEAT1 expression levels with clinicopathologic characteristics of CRC. As shown in Table 2, levels of NEAT1 were positively associated with CEA level (p = 0.005), tumor size (p = 0.041), presence of distant metastasis (p = 0.037) and TNM stage (p = 0.045). In contrast, no significant associations were found between NEAT1 expression and other clinical features, including age, sex, tumor location and lymphatic metastasis.

To directly identify the risk factors associated with OS in CRC patients, univariate and multivariate analysis were performed to confirm that NEAT1 represents an independent risk factor for poor prognosis. Univariate Cox regression analysis showed that NEAT1 expression level, CEA level and TNM stage were significantly associated with OS (Table 3). Furthermore, multivariate Cox regression analysis confirmed that NEAT1 expression level and TNM stage were independent predictors of OS in patients with CRC (Table 3). These data indicated that high expression of NEAT1 may be a predictor for the diagnosis and prognosis in colorectal cancer patients.

3.3. LncRNA NEAT1 promotes the proliferation and invasion of CRC cells

Our findings have indicated that NEAT1 is up-regulated and can serve as an independent overall survival predictor in CRC patients. In further experiments, we synthesized and transfected siRNAs targeting NEAT1 or plasmids (pcDNA3.1-NEAT1) to down-regulate or up-regulate NEAT1 expression levels to perform loss-of-function or gain-of-function studies in colorectal cell lines. The NEAT1 relative expression level in CRC cells is shown in Supplementary Fig. 1. The transfection efficiency was confirmed by RT-qPCR (Fig. 2A and B). CCK-8 assays and transwell assays showed that the proliferation and invasion capacities of CRC cells (HCT116, SW620, HT-29 and SW480 cells) transfected with siRNA-NEAT1 were significantly lower than those of the control group (Fig. 2C and D, 2H, Supplementary Fig. 2), while the proliferation and invasion capacities of HT-29 and SW480 cells transfected with pcDNA3.1-NEAT1 were significantly enhanced (Fig. 2E–G). These results suggest that NEAT1 promotes the proliferation and invasion of CRC cells.

3.4. LncRNA NEAT1 directly interacts with miR-34a in CRC

Recent studies have demonstrated that NEAT1 can act as competitive endogenous RNAs (ceRNAs) for miRNAs, regulating their biological functions [8,10]. We performed a search for miRNAs that have complementary base pairing with NEAT1 using the online software program starBase (http://starbase.sysu.edu.cn). Our search results identified that 36 miRNAs formed complementary base pairing with NEAT1 (scores > 1000, Supplementary Table S1). Subsequently, only four miRNAs (miR-34a, miR-342–3p, miR-193b-3p and miR-181d-5p)

Table 3
Univariate and multivariate analyses of prognostic parameters for survival in 100 colorectal cancer patients.

Prognostic parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Expression of NEAT1 (low vs. high)	1.918	1.098–3.349	**0.022**	1.905	1.071–3.389	**0.028**
Age (< 65 vs. ≥65)	1.511	0.870–2.622	0.143	–	–	–
Sex (male vs. female)	1.145	0.662–1.981	0.629	–	–	–
Tumor Size (≤5 cm vs. > 5 cm)	2.258	1.250–4.079	**0.007**	1.667	0.884–3.145	0.115
CEA level (≤5 ng/ml vs. > 5 ng/ml)	1.597	0.913–2.792	0.101	–	–	–
Tumor location (rectum vs. colon)	1.186	0.686–2.050	0.542	–	–	–
TNM stage (I vs. II vs. III vs. IV)	2.261	1.786–3.392	**0.000**	2.195	1.565–3.079	**0.000**

HR: Hazard ratio; CI: Confidence interval. The bold number represents the *P*-values with significant differences.

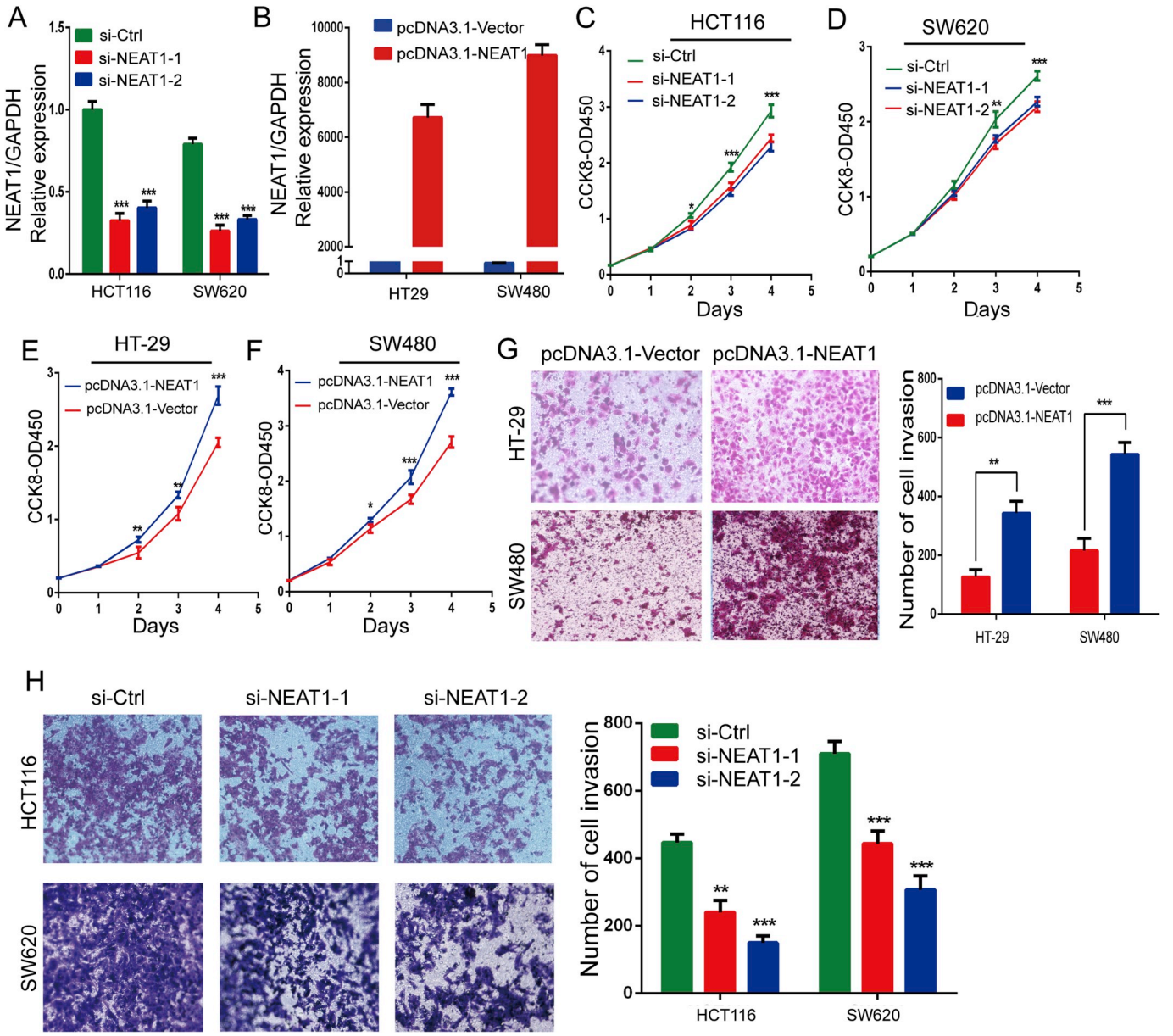

Fig. 2. NEAT1 promotes cell proliferation and invasion in CRC cells. (A–B): NEAT1 knockdown or overexpression efficiency was confirmed by RT-qPCR in CRC cells. **(C–F):** Effects of NEAT1 knockdown or overexpression on proliferation was evaluated by CCK-8 assay. **(G–H):** Effects of NEAT1 knockdown or overexpression on invasion was evaluated by transwell assay. Results shown are the mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001) of triplicate determination from three independent experiments.

showed abnormal expression in CRC tissues in the bioinformatics software (starBase 2.0). By RT-qPCR analysis, we found that miR-34a and miR-193b-3p were significantly down-regulated, whereas miR-342–3p and miR-181d-5p were up-regulated in CRC (Fig. 3A and B, Supplementary Fig. 3). Subsequently, miR-34a expression was significantly reduced by treatment with pcDNA3.1-NEAT1 and remarkably increased by treatment with siRNA-NEAT1 in CRC cells, whereas miR-193b-3p showed no changes (Fig. 3C and D). We further constructed two luciferase reporter plasmids encoding a firefly luciferase transcript with either wild-type or mutant NEAT1 (Fig. 3E). Overexpression of miR-34a significantly inhibited the luciferase reporter activity of the wild-type NEAT1, whereas no significant difference was observed with the overexpression of miR-193b-3p (Fig. 3F). In addition, we observed a negative correlation between NAET1 and miR-34a expression ($p = 0.026$, $R = -0.4047$), but not miR-193b-3p expression ($p = 0.825$, $R = -0.0419$), in CRC tissues via correlation analysis (Fig. 3G). Taken together, we speculated that lncRNA NEAT1 may exert an effect on deregulation of miR-34a, acting as a ceRNA in colorectal cancer.

3.5. LncRNA NEAT1 regulates CRC cell proliferation and invasion through miR-34a

Our previous results indicated that NEAT1 could act as an oncogene in CRC progression and that NEAT1 was a direct target of miR-34a. We next investigated whether NEAT1 exerts biological functions in CRC cells through miR-34a. MiR-34a mimics were transfected into CRC cells stably co-transfected with NEAT1 cDNA or NEAT1-Mut cDNA (mutant miR-34a binding site) or a negative control. CCK-8 assays revealed that the promotion of cell proliferation induced by NEAT1 cDNA was significantly reversed by treatment with miR-34a mimics (Fig. 4A and B). In addition, overexpression of miR-34a inhibits the enhanced ability of CRC cells to invade caused by NEAT1 cDNA (Fig. 4C and D). Furthermore, we observed that NEAT1-Mut cDNA did not affect cell

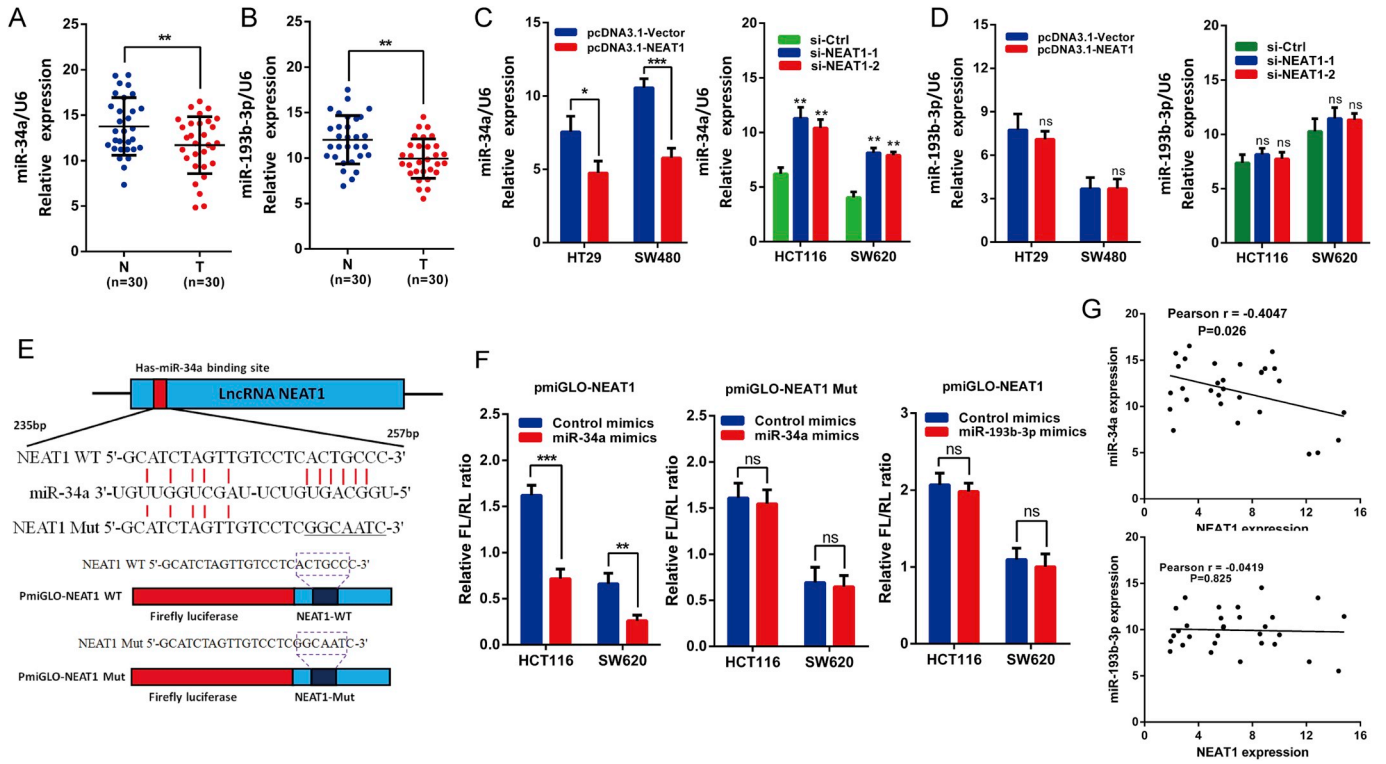

Fig. 3. NEAT1 is physically targeting to miR-34a. (A–B): the level of miR-34a and miR-193b-3p in 30 pairs of CRC tissues was detected by RT-qPCR and normalized by an endogenous control, U6 RNA. **(C–D):** the level of miR-34a and miR-193b-3p was detected in the presence of pcDNA3.1-NEAT1 or siRNA-NEAT1 by RT-qPCR. **(E):** The bioinformatic software was predicted miRNAs of potentially target NEAT1. **(F):** Luciferase activity in HCT116 and SW620 co-transfected with miR-34a or miR-193b-3p and luciferase reporters containing NEAT1 or mutant transcript. **(G):** Negative correlation between NEAT1 and miR-34a, but not miR-193b-3p in 30 CRC tissues. Results shown are the mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001) of triplicate determination from three independent experiments.

proliferation and invasion compared with control group (Fig. 4E–H). And CCK8 or transwell assays results showed that the decreased proliferative or invasive ability of HT-29 cells and SW480 cells induced by miR-34a mimics did not reversed by treatment with NEAT1-Mut cDNA (Fig. 4E–H). Taken together, these data suggest that NEAT1 promotes CRC cell proliferation and invasion through miR-34a.

3.6. LncRNA NEAT1 regulates SIRT1 expression via competitive binding with miR-34a

Increasing evidence revealed that the human SIRT1 acts as an oncogene in colorectal cancers [14,15], and miR-34a functions as a tumor suppressor by targeting SIRT1 in CRC cells [16]. Therefore, we used four bioinformatic software (DIANA-microT (http://www.microrna.gr/microT), RNA22 (http://cbcsrv.watson.ibm.com/rna22.html), PicTar (http://www.pictar.org/) and microRNA.org (http://www.microrna.org/)) to explore potential downstream target genes. As a result, SIRT1, MYCN, JAG1, ZDHHC17 and ACSL1 were identified as candidates. Then, we detected the candidate mRNA expression in the presence of miR-34a mimics or miR-34a inhibitors or siRNA-NEAT1 of CRC cells. The resulted showed that SIRT1, MYCN and ACSL1 mRNA expression was significantly reduced by treatment of miR-34a mimics, and remarkably increased by treatment of miR-34a inhibitors (Supplementary Fig. 4). Furthermore, we found siRNA-NEAT1 could decrease SIRT1 levels, not the MYCN and ACSL1 expression in HCT116 and SW620 cells (Supplementary Fig. 4). Then, the SIRT1 3′UTR fragment containing the wild-type or mutant miR-34a-binding site was inserted downstream of the luciferase open reading frame (Fig. 5A). Our results showed that SIRT1 3′UTR is a direct target of miR-34a in CRC cells, which was validated by luciferase reporter activity in our studies (Fig. 5B). We further showed that miR-34a exerts its tumor-suppressive effects by regulating SIRT1 in CRC cells (Supplementary Fig. 4).

To determine whether NEAT1 promotes CRC progression in a miR-34a/SIRT1-dependent manner, we performed RT-qPCR and Western blot analysis. Our results showed that NEAT1 knockdown decreased SIRT1 levels, while miR-34a inhibition rescued this reduction in HCT116 and SW620 cells (Fig. 5C) Conversely, overexpression of NEAT1 increased SIRT1 expression, whereas treatment with miR-34a mimics abrogated the NEAT1-induced up-regulation of SIRT1 (Fig. 5D). Furthermore, we assayed luciferase activity after transfection of a luciferase plasmid containing the SIRT1 3'-UTR in HCT116 and SW620 cells. NEAT1 depletion decreased the luciferase activity of pmirGLO-SIRT1-3'-UTR, and this reduction was restored by inhibition of miR-34a. Reciprocally, NEAT1 overexpression increased the luciferase activity of pmirGLO-SIRT1-3'-UTR, while forced expression of miR-34a mimics abolished this up-regulation (Fig. 5E and F). Finally, we observed that SIRT1 expression was positively correlated with NEAT1 expression ($R = 0.501$, $p = 0.005$) and negatively correlated with miR-34a expression ($R = -0.502$, $p = 0.005$) in CRC tissues (Fig. 5G and H). The above results collectively suggest that NEAT1 competitively binds to miR-34a to promote SIRT1 expression, thereby promoting the initiation and progression of CRC.

3.7. The lncRNA NEAT1 promotes malignant CRC progression via the Wnt/β-catenin signaling pathway

Many studies have reported that SIRT1 plays crucial roles in various cell processes via activation of the Wnt/β-catenin signaling pathway [17,18], which is consistent with our findings in HT-29 and SW480 (Fig. 6A). Thus, to further explore the potential mechanism involved in the NEAT1-associated malignant progression of CRC, we determined the expression levels of key members of the Wnt/β-catenin signaling pathway. Western blot analysis revealed that the levels of β-catenin and

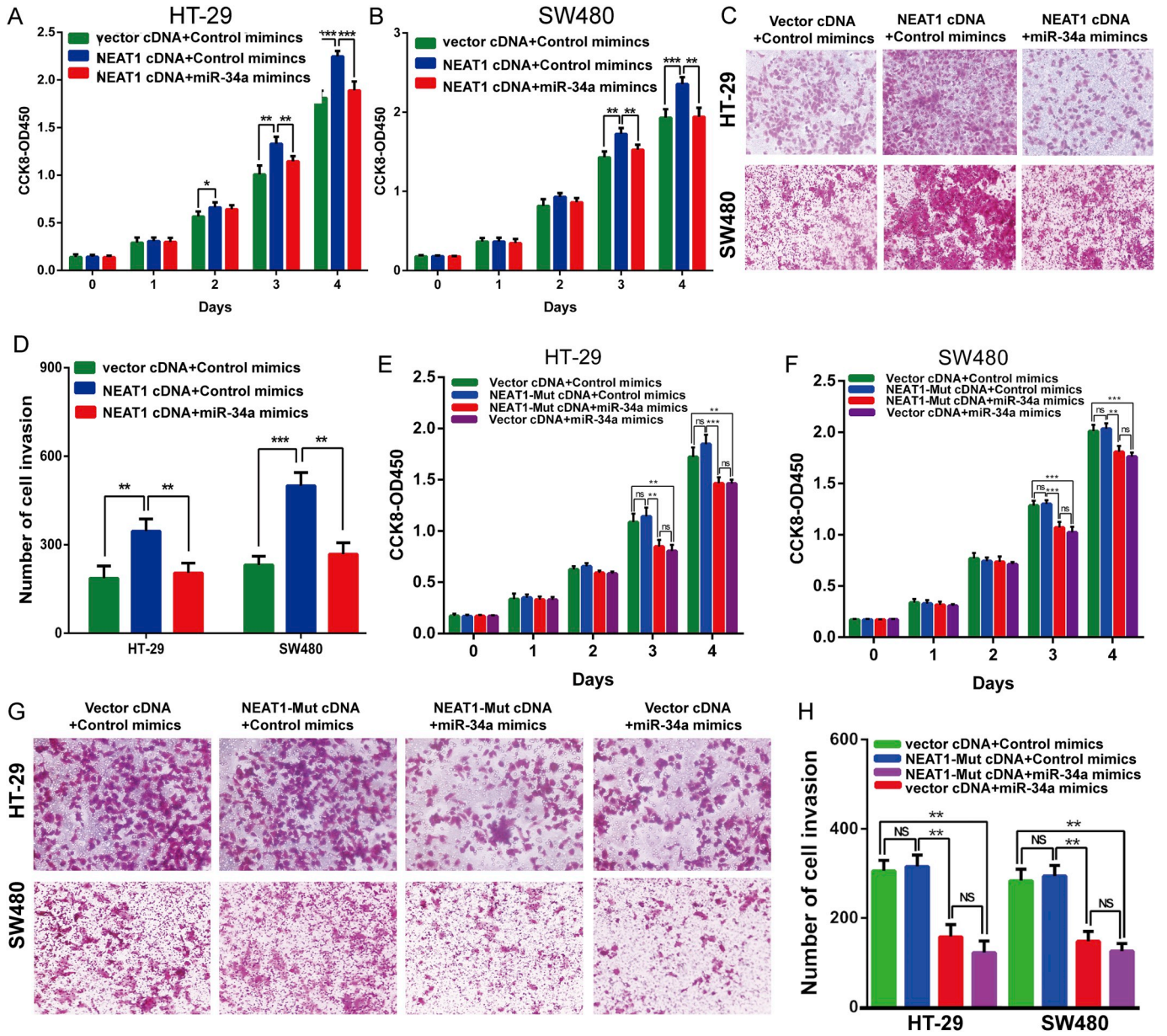

Fig. 4. MiR-34a mimics restored the promoted effects on CRC cells induced by NEAT1 overexpression. (A–B): The promoted effect of NEAT1 overexpression on cell proliferation in HT-29 and SW480 cells could be restored by miR-34a mimics by CCK-8 assay. **(C–D):** Transwell assay showed that NEAT1 overexpression induced cell invasion, which was largely abolished by miR-34a mimics. **(E–F):** the decreased proliferative or invasive ability of HT-29 cells and SW480 cells induced by miR-34a mimics did not reversed by treatment with NEAT1-Mut cDNA. Results shown are the mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001) of triplicate determination from three independent experiments.

several Wnt/β-catenin pathway transcriptional targets, such as c-myc and cyclin D1, were significantly reduced, whereas E-cadherin expression was markedly augmented when NEAT1 was knocked down in CRC cells (Fig. 6B). Furthermore, overexpression of NEAT1 caused the opposite effect (Fig. 6C). In addition, si-SIRT1 treatment in CRC cells could interfere with the increase in Wnt/β-catenin pathway expression caused by NEAT1 cDNA treatment (Fig. 6D). These data therefore suggest that NEAT1 may promote colorectal cancer cell progression via the SIRT1-Wnt/β-catenin signaling pathway axis.

3.8. The lncRNA NEAT1 promotes CRC growth and metastasis in vivo by inhibiting the miR-34a/SIRT1/Wnt/β-catenin axis

To validate the effects of NEAT1 on tumorigenesis and metastasis *in vivo*, HT29-NEAT1-cDNA cells or appropriate control cells were subcutaneously injected into nude mice. MiR-34a agomir was subsequently injected directly into the implanted tumors five days after injection and every five days thereafter, which significantly decreased tumor growth *in vivo* (Supplementary Fig. 5). We observed that tumor size, tumor weight and incidence of liver metastasis were significantly increased in HT29-NEAT1-cDNA cells compared with controls (Fig. 7A–E). Interestingly, miR-34a agomir-treated xenograft tumors from HT29-NEAT1-cDNA cells showed decreased tumor growth and rates of liver metastasis (Fig. 7A–E). Furthermore, RT-qPCR and Western blot assays showed that up-regulation of NEAT1 promoted the expression of SIRT1 and Wnt/β-catenin signaling pathway members, whereas miR-34a agomir treatment decreased the levels of SIRT1 and Wnt/β-catenin signaling pathway members in xenograft tumors generated from HT29-NEAT1-cDNA cells (Fig. 7F and G). Taken together, these results demonstrate that NEAT1 plays a crucial role in CRC

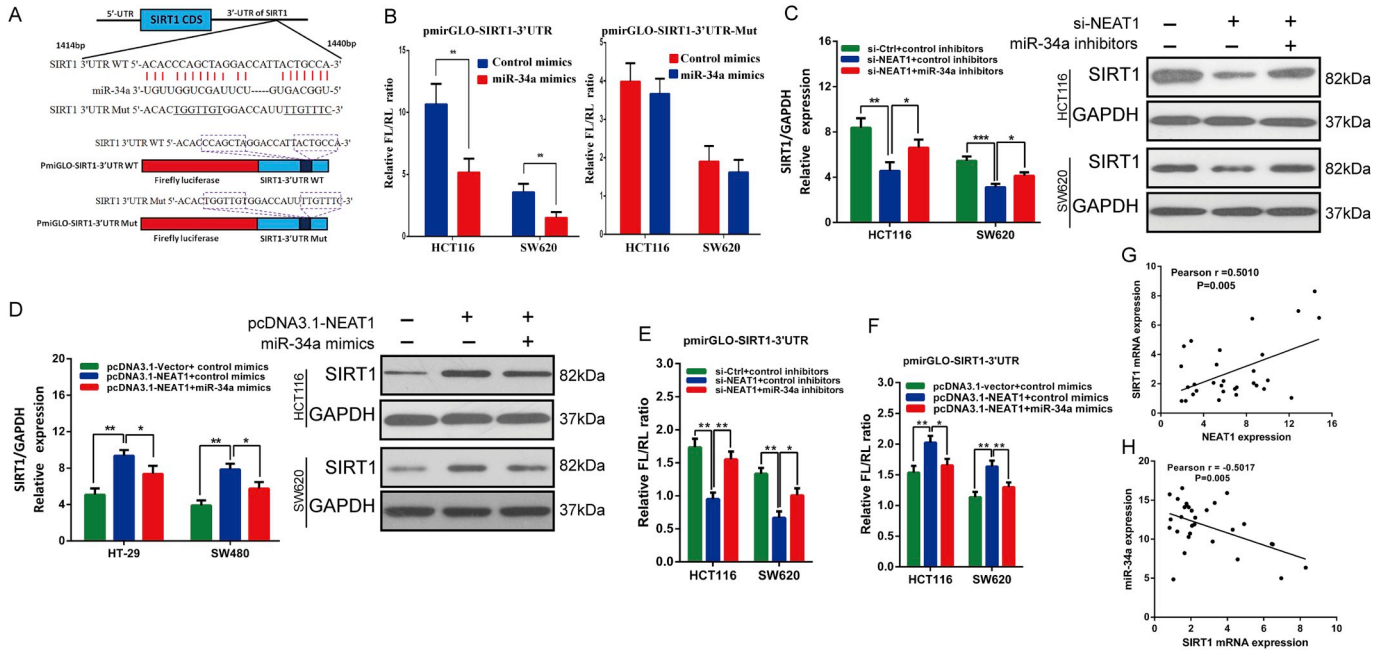

Fig. 5. NEAT1 modulates SIRT1 expression by competitively binding miR-34a. (A): Putative SIRT1 base pairing with miR-34a as identified by the bioinformatic software. **(B):** the luciferase activity was detected after co-transfection of SIRT1 3'-UTR or its mutant form, miR-34a vector or control vector into CRC cells. **(C):** NEAT1 knockdown decreased SIRT1 while miR-34a inhibitors rescued the reduction in HCT116 and SW620 cells by the RT-qPCR and western blotting. **(D):** overexpression of NEAT1 increased SIRT1, whereas miR-34a mimics abrogated NEAT1-induced up-regulation of SIRT1 in HT-29 and SW480 cells by the RT-qPCR and western blotting. **(E):** NEAT1 depletion decreased the luciferase activity of pmirGLO-SIRT1-3'-UTR, which was restored by inhibition of miR-34a. **(F):** NEAT1 overexpression increased the luciferase activity of pmirGLO-SIRT1-3'-UTR while forced expression of miR-34a abolished this up-regulation. **(G):** Positive correlation between NEAT1 and SIRT1 expression in 30 CRC tissues. **(H):** Negative correlation between SIRT1 and miR-34a in 30 CRC tissues. Results shown are the mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001) of triplicate determination from three independent experiments.

growth and metastasis through inhibition of the miR-34a/SIRT1/Wnt/β-catenin axis (Fig. 8).

4. Discussion

This report is the first direct investigation of the function and mechanism of action of the lncRNA NEAT1 in colorectal cancer. First, we identified that NEAT1 expression was frequently up-regulated in colorectal cancer, which was correlated with poorer survival rates of colorectal cancer patients. Next, we identified NEAT1 as an oncogenic lncRNA that promoted cell growth and metastasis of colorectal cancer cells both *in vitro* and *in vivo,* and down-regulation of NEAT1 had the opposite effect. Mechanistically, NEAT1 promotes CRC progression by functioning as a ceRNA to repress the miR-34a/SIRT1 feedback loop and subsequent activation of the Wnt signaling pathway.

Accumulating evidence has indicated that NEAT1 can play critical roles in the tumor progression of many malignant cancers in humans [8–10]. For example, Wang et al. reported that NEAT1 expression was up-regulated in gastric tissues. Moreover, knockdown of NEAT1 exerted tumor-suppressive functions by inhibiting cell proliferation, migration and invasion [19]. Sun et al. demonstrated that high NEAT1 expression predicted poor prognosis in patients with non-small-cell lung cancer (NSCLC), and NEAT1 promoted malignant progression of NSCLC through regulation of the miR-377–3p/E2F3 axis [10]. Furthermore, Li et al. illustrated that NEAT1 expression in clinical colorectal cancer was an independent prognostic factor of disease-free and overall survival for affected patients [20], which is contrast to the Zhang et al. [21] and Peng et al. studies [22]. However, Xiong et al. revealed that the expression levels of NEAT1 in CRC tissues were not significantly different from those of NEAT1 in non-tumor samples [23]. Wu et al. found high expression of NEAT1_v1, not the NEAT1_v2, was correlated with worse overall survival in CRC patients [24]. Therefore, the role of NEAT1 in CRC cells needs to be further studied.

In this study, we found that NEAT1 expression was markedly increased in CRC tissues and cells, consistent with findings from Li et al. [20]. Moreover, the expression of NEAT1 was positively associated with the aggressiveness and recurrence of CRC. Thus, it can be concluded that NEAT1 acts as an oncogenic gene or risk factor in CRC tumorigenesis, which is consistent with previous studies [21]. Although lncRNAs don't have any protein-coding capacity, they constitute a substantial proportion of the transcriptome and can modulate genetic expression via immediate or indirect regulation [5,6]. Through *in vitro* and *in vivo* experiments, we found that NEAT1 increased CRC cell viability and invasion capacity, indicating the oncogenic role of NEAT1 in colorectal cancer tumorigenesis.

Recent studies indicate that NEAT1 is an important lncRNA for the integrity of nuclear paraspeckle substructure, which contain ribonucleoprote in complexes formed around NEAT1 [25]. Although NEAT1 was mainly located in the nucleus with a small amount in the cytoplasm, NEAT1 containing miRNA-binding sites could regulate and communicate with mRNAs by competing specifically for shared miRNAs, thus acting as ceRNAs [8,10,19,26]. For example, Qian et al. demonstrated that NEAT1 was a potential oncogene in breast and that NEAT1 could accelerate cell growth and metastasis by sponging miR-101 [8]. Li et al. also showed that NEAT1 functioned as a ceRNA by sponging miR-214 in thyroid carcinoma, promoting cell survival, migration and invasion [26]. With the help of online bioinformatics programs, we identified that miR-34a, acting as a connecting carrier, targets both NEAT1 and the 3'-UTR of SIRT1. Luciferase reporter assays revealed that both NEAT1 and SIRT1 are physically associated with miR-34a. In addition, our results showed that ectopic NEAT1 expression led to repression of miR-34a expression, which rescued SIRT1 expression in CRC cells. Taken together, we demonstrate that NEAT1 can directly interact with and down-regulate miR-34a, resulting in

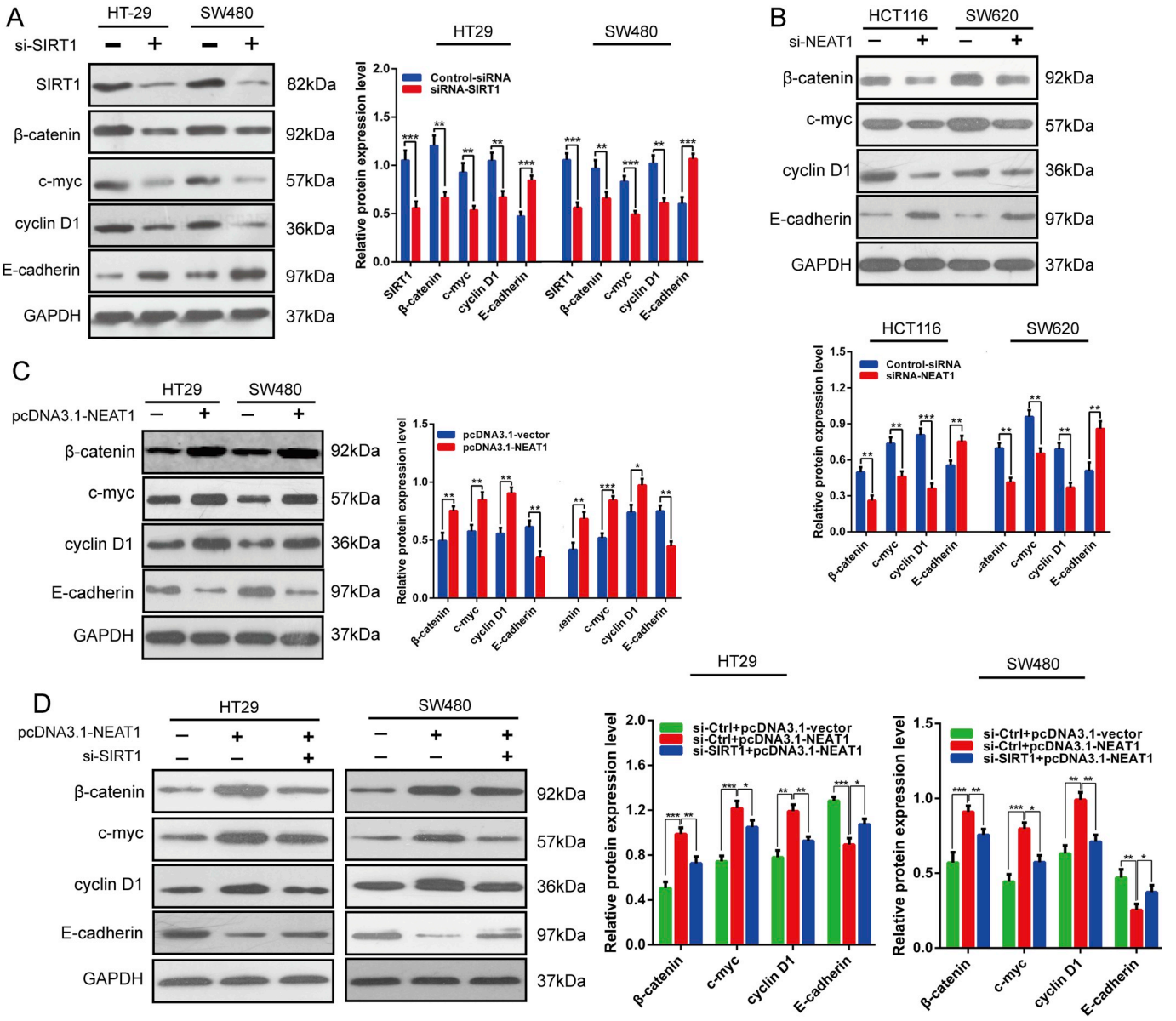

Fig. 6. NEAT1 positively regulated the Wnt/β-catenin signaling pathway through SIRT1. (A): Expression of β-catenin, c-myc, cyclin D1, E-cadherin proteins were determined in HT-29 and SW480 cells transfected with siRNA-SIRT1. **(B):** SIRT1 inhibition decreased the protein expression of β-catenin, c-myc, cyclin D1, while E-cadherin protein expression was augmented in HCT116 and SW620 cells by Western blot. **(C):** SIRT1 overexpression increased the protein expression of β-catenin, c-myc, cyclin D1, while E-cadherin protein expression was decreased in HT-29 and SW480 cells by Western blot. **(D):** The promoted effect of NEAT1 overexpression on the protein expression levels of Wnt/β-catenin signaling pathway in HT-29 and SW480 cells could be restored by SIRT1 inhibition. Results shown are the mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001) of triplicate determination from three independent experiments.

increased SIRT1 expression. This constitutes the identification of a competitive endogenous RNA network in colorectal cancer.

Several lncRNAs have been suggested to enhance or inhibit the Wnt/β-catenin signaling pathway's role in regulating malignant colorectal cancer processes [27,28]. Previous studies also demonstrated that NET1 could regulate Wnt/β-catenin signaling pathway in various tumor processes [21,29,30]. Geng et al. found overexpression of NEAT1 could promote cell proliferation, migration and invasion by activating the Wnt/β-catenin signaling pathway in multiple myeloma [29]. Chen et al. illuminated that NEAT1 promoted the Wnt/β-catenin signaling pathway activity through regulated the levels of ICAT, GSK3B, and Axin2 in glioblastoma [30]. Peng et al. showed that NEAT1 displays oncogenic activity is related with the modulation of Akt pathway, which significantly affecting Wnt/β-catenin signaling pathway [22,27]. However, the association between the ceRNA of NEAT1 and the Wnt/β-catenin signaling pathway in CRC has not previously been studied. Our research revealed that knockdown of NEAT1 significantly reduces the expression levels of tumor-related genes (e.g., β-catenin, c-myc, and cyclin-D1) in the Wnt/β-catenin signaling pathway in CRC cells, and overexpression of NEAT1 has the opposite effect.

SIRT1, participating in the initiation and progression of many types of human cancers, play crucial roles in activation of the Wnt/β-catenin signaling pathway [17,18]. SIRT1 expression stimulates phosphorylation of β-catenin at S675 through stimulating phosphorylation of PKAα, which is critical for the βTrCP-mediated ubiquitination and subsequent degradation of β-catenin [18]. Yu et al. also demonstrated that down-regulation of the lncRNA CRNDE remarkably activates the Wnt/β-catenin signaling pathway, promoting cell proliferation and metastasis of CRC through the miR-217/TCF7L2 axis [31]. Our studies also expounded that NEAT1 promotes CRC progression by functioning as a

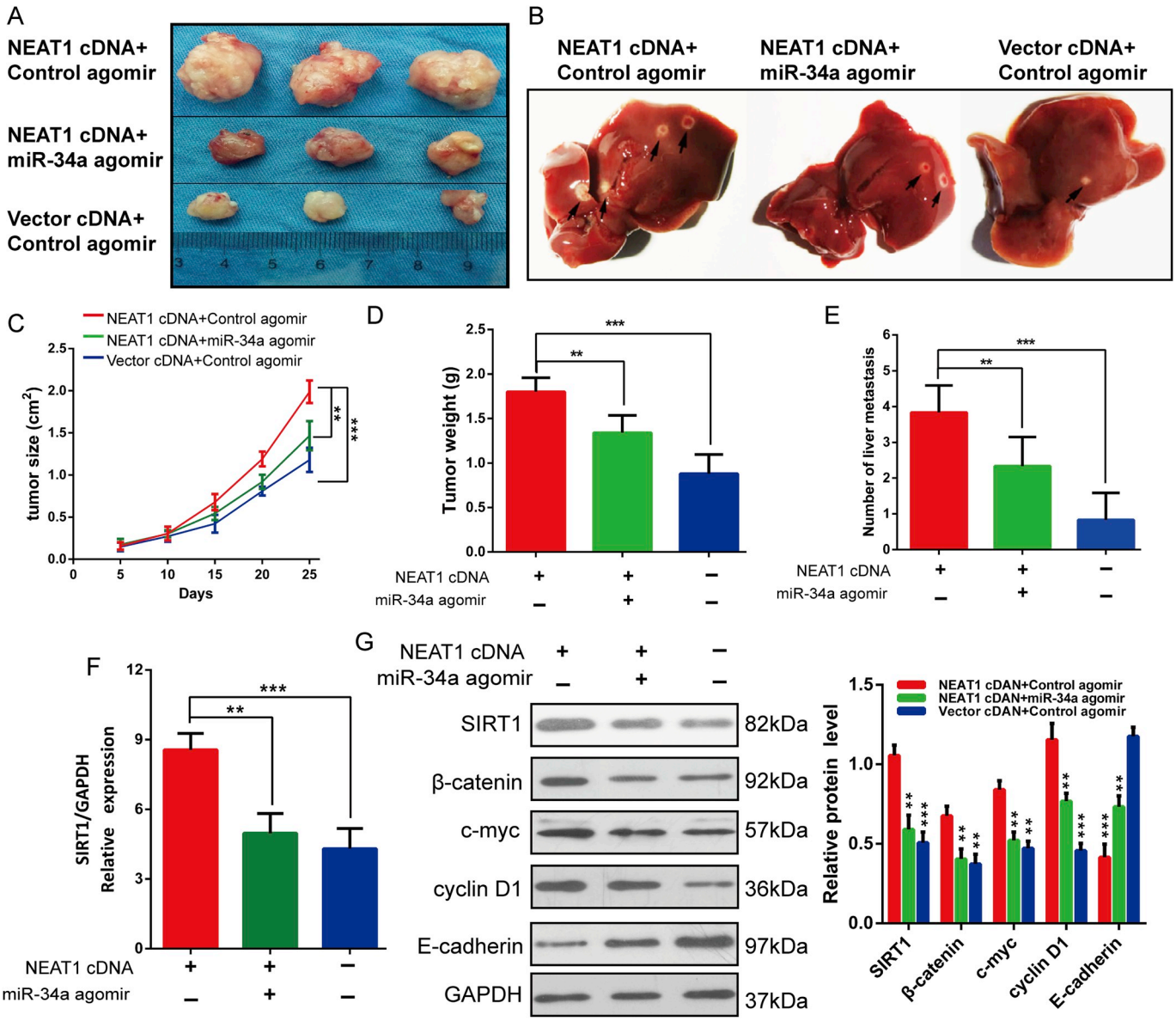

Fig. 7. NEAT1 promotes CRC growth and metastasis *in vivo* by inhibiting miR-34a/SIRT1/Wnt/β-catenin axis. (A–E): The promoted effect of NEAT1 overexpression on tumor growth, tumor size, tumor weight and metastasis in colorectal cancer could be restored by miR-34a. **(F–G):** The promoted effect of NEAT1 overexpression on mRNA and protein levels of SIRT1, and protein levels Wnt/β-catenin signaling pathway in colorectal cancer could be restored by miR-34a.

ceRNA to repress the miR-34a/SIRT1 feedback loop. Furthermore, down-regulation of SIRT1 expression could restore the repression of β-catenin, c-myc, and cyclin-D1 expression induced by NEAT1 overexpression. However, one limitation should not be ignored in this study: whether the NEAT1-mediated β-catenin accumulation could stimulate phosphorylation of PKAα in CRC cells, need our further studies.

In conclusion, our results illustrate that NEAT1 functions as an oncogenic lncRNA to facilitate the tumorigenesis and progression of colorectal cancer by competitively binding miR-34a, increasing SIRT1 expression and enhancing the Wnt/β-catenin signaling pathway. The present results elucidate a potential mechanism underlying the tumor-oncogenic role of NEAT1 in colorectal cancer and indicate that NEAT1 could serve as a useful marker and potential therapeutic target in colorectal cancer.

Conflicts of interest

The authors declare that they have no competing financial interests to disclose.

Abbreviations

lncRNAs, long noncoding RNAs; NEAT1, nuclear enriched abundant transcript 1; CRC, colorectal cancer; RT-qPCR, real-time quantitative PCR; FBS, fetal bovine serum; ceRNAs, competitive endogenous RNAs; OS, overall survival; DFS, disease-free survival; NSCLC, non-small-cell lung cancer.

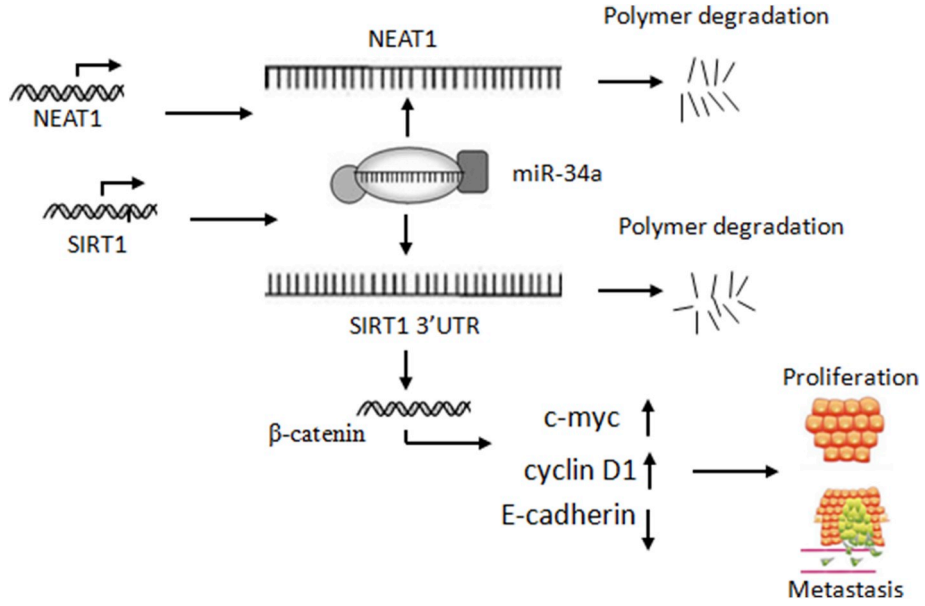

Fig. 8. Diagram depicting the regulation mechanism of NEAT1 in the tumorigenesis of colorectal cancer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.10.002.

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