Original Paper

The LncRNA NEAT1 Accelerates Lung Adenocarcinoma Deterioration and Binds to Mir-193a-3p as a Competitive Endogenous RNA

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Key Words
Neat1 • LUAD • MiR-193a-3p • Biological function • CeRNA

Abstract
Background/Aims: Long noncoding RNAs (lncRNAs) contribute to the development of multiple malignant tumors. Here, we focused on the biological function and underlying molecular mechanism of an lncRNA, nuclear-enriched abundant transcript 1 (NEAT1), in lung adenocarcinoma (LUAD). ***Methods:*** *In vitro* experiments were conducted to determine the biological effects of NEAT1 in LUAD cells. A luciferase activity reporter assay was performed to corroborate the interaction between NEAT1 and miR-193a-3p. Data from Gene Expression Omnibus (GEO), Oncomine, The Cancer Genome Atlas (TCGA), and our in-house reverse transcription quantitative PCR (RT-qPCR) were combined to examine the expression of NEAT1 and miR-193a-3p in LUAD. To further explore the regulatory mechanism of NEAT1, we searched for putative target genes of miR-193a-3p from 12 online prediction databases and determined genes positively correlated with NEAT1 as candidate targets. Furthermore, we analyzed the expression of these selected genes using data from TCGA. ***Results:*** *In vitro* experiments showed that knockdown of NEAT1 in LUAD cells markedly restrained cell proliferation, invasion, and migration and stimulated cell apoptosis. The dual-luciferase reporter assay demonstrated that miR-193a-3p directly targeted NEAT1 at its 3'-UTR. We then detected NEAT1 and miR-193a-3p in LUAD cells and normal lung epithelial cells and discovered high expression of NEAT1 and low expression of miR-193a-3p in LUAD cell lines. Simultaneously, the pooled results from the GEO, Oncomine, TCGA, and in-house RT-qPCR showed that the NEAT1 expression increased while the miR-193a-3p expression decreased in LUAD tissues versus normal lung tissues. Furthermore, the USF1 gene was not only upregulated in LUAD, but also positively correlated with NEAT1, suggesting that NEAT1 may function as a ceRNA to sponge miR-193a-
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3p and abrogate the inhibitory effect of miR-193a-3p on USF1. ***Conclusions:*** Our findings indicate that NEAT1 plays important roles in the occurrence and progression of LUAD. It may exert its role by acting as a ceRNA to regulate miR-193a-3p.

Introduction

Lung cancer is the main cause of oncogenic mortality in both men and women worldwide [1, 2]. Non-small cell lung carcinoma (NSCLC), the most prevalent form of lung cancer, includes three histopathological subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [3]. Lung adenocarcinoma (LUAD) is the most common variant, accounting for nearly 40% of lung cancer cases [3, 4]. Despite improved understanding of oncogenic drivers, the potential molecular mechanism of LUAD remains unclear. Thus, investigation of the possible mechanism of LUAD pathogenesis is an urgent and challenging task.

Increasing numbers of studies have concentrated on long noncoding RNAs (lncRNAs), which may be cell-type specific RNAs that contribute to tumor onset and progression via multiple ways, including cell cycle regulation, transcriptional/post-transcriptional regulation, and DNA methylation [5-8]. Nuclear-enriched abundant transcript 1 (NEAT1), a structural component of paraspeckle, plays critical roles in several malignancies, including lung cancer. Our research group has previously examined the expression of NEAT1 in NSCLC via reverse transcription quantitative PCR (RT-qPCR) [9]. The results indicated that NEAT1 was upregulated in NSCLC compared with adjacent non-tumorous tissues, with overexpressed NEAT1 strongly associated with several malignant clinical phenotypes, such as lymphatic metastasis, TNM stage, and vascular invasion. A study by Sun et al [10]. showed that NEAT1 played key roles in NSCLC by promoting the Wnt/β-catenin pathway. Accumulating studies have reported that lncRNAs, including NEAT1, exert roles in tumors by functioning as miRNA sponges or competing endogenous RNAs (ceRNAs) to interact with miRNAs. In NSCLC, NEAT1 has been demonstrated to competitively bind to miRNAs, such as miR-181a-5p and miR-98, and subsequently upregulate their target genes [11, 12]. Sun et al [13]. discovered that NEAT1 accelerated NSCLC development by acting as a ceRNA for miR-377-3p and antagonizing the inhibition of miR-377-3p on its target gene E2F3, a critical oncogene in NSCLC carcinogenesis. Similarly, the impact of NEAT1 on the miR-377-3p/E2F3 axis in NSCLC was recapitulated in a study conducted by Zhang et al [14].. However, the understanding of the underlying role and functional mechanism of NEAT1 in LUAD is still limited. We therefore carried out this study to elucidate the possible regulatory mechanism of NEAT1 in LUAD.

In the present study, we first performed *in vitro* experiments to investigate the biological function of NEAT1 in LUAD. A luciferase activity assay was then conducted to verify the interaction between NEAT1 and miR-193a-3p. Microarray and RNA-seq data were mined to validate the expression of NEAT1 and miR-193a-3p in LUAD. Furthermore, the potential targets of miR-193a-3p were predicted by online tools, and bioinformatics analysis was subsequently performed to explore the active mechanism of NEAT1 in LUAD.

Materials and Methods

Cell culture and transfection

Four LUAD cell lines (H1299, A549, HCC827, and Calu3), one human renal epithelial cell line (293T), and one normal lung epithelial cell line (BEAS-2B) were purchased from ScienCell Research Laboratories (Carlsbad, CA) and cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel) and 1% penicillin/streptomycin (Invitrogen). Cells were transfected with mock control, blank control, scrambled siRNAs, and NEAT1 siRNAs for *in vitro* experiments according to the manufacturer's instructions. After 48 hours, the transfection efficiency was verified via RT-qPCR.

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:905-918

DOI: 10.1159/000491958
Published online: July 23, 2018

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Expression of NEAT1 and miR-193a-3p by RT-qPCR

Cellular RNA was isolated using TRNzol reagent (TIANGEN, Beijing, China) and converted to cDNA using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kits (TransGen Biotech, Beijing, China). Subsequently, RT-qPCR was carried out with a CFX96TM Touch PCR system (Bio-Rad Laboratories, Hercules, CA). NEAT1 primer sequences (forward 5'-TTACCAGCTTCCTCCTGGTG-3', reverse 5'-TCTGCTGCGTATGCAAGTCT-3') and the miR-193a-3p forward primer sequence (5'-TAACTGGCCTACAAAGTCCCA-3') were synthesized by Sangon Biotech (Shanghai, China). HS-ACTB (Sangon Biotech) served as the endogenous control. Relative NEAT1 and miR-193a-3p abundances were determined with the $2^{-\Delta CT}$ method [15].

Biological effects of NEAT1 on LUAD cells in cell functional experiments

We selected cell lines for further *in vitro* experiments based on the aforementioned RT-qPCR results. Cell viability and proliferation were detected with fluorometric detection of resorufin (CellTiter-Blue Cell Viability Assay, G8080, Promega, Madison, WI) and colorimetric tetrazolium (MTS) assay (CellTiter96 AQueous One Solution Cell Proliferation Assay G3580, Promega), respectively. Cell apoptosis was examined with Hoechst 33342 and propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) double-fluorescent chromatin staining and the Apo-ONE® Homogeneous Caspase-3/7 Assay (G7790, Promega). Two reviewers independently counted the viable and apoptotic cells in 10 fields of each well under a 200× magnification microscope after Hoechst 33342/PI double-fluorescent chromatin staining at 96 hours. A wound-healing assay was conducted to monitor cell migration. Photographs of the wound were obtained at 0, 12, 24, and 48 hours to observe cell migration and calculate the wound width. The wound widths at 12, 24, and 48 hours were compared with the wound width of the blank control at 0 hours. A Transwell assay was performed to detect the effects of NEAT1 on LUAD cell invasion. Cells were independently counted in five random fields under a microscope by two investigators. All *in vitro* experiments were conducted as reported in preceding studies [16-19].

Validation of NEAT1 and miR-193a-3p binding based on luciferase activity

To corroborate whether NEAT1 directly binds to miR-193a-3p, a human renal cell line 293T was chosen for the luciferase activity assay due to its high transfection efficiency. The wild-type (WT) NEAT1 3'-UTR binding site of miR-193a-3p was subcloned into pGL3 Basic vector (Promega). Similarly, the WT TRAF6 3'-UTR binding site of miR-146 was also cloned into pGL3 Basic vector (Promega) as a positive control [20, 21]. WT NEAT1-3'-UTR or WT TRAF6-3'-UTR vector and miRNA mimic or negative control (NC) plasmid were then co-transfected into 293T cells. After 48 hours, the luciferase activity was determined with the Dual-Luciferase® Reporter Assay System (Promega) in accordance with the manufacturer's protocols.

Statistical analysis

All experiments were performed in triplicate, and continuous variable results are presented as the mean ± standard deviation (SD). A Student's t test or one-way analysis of variance was used for comparisons between groups. All statistical analyses were carried out using SPSS 22.0 (IBM, Armonk, NY) and GraphPad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA). $P<0.05$ indicated statistical significance.

Clinical roles of NEAT1 and miR-193a-3p based on meta-analyses

To investigate the expression and correlation of NEAT1 and miR-193a-3p in LUAD, we performed a meta-analysis based on data from Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/), Oncomine (https://www.oncomine.org/resource/main.html), The Cancer Genome Atlas (TCGA; https://cancergenome.nih.gov/), and our in-house RT-qPCR. The following search strategies were used to retrieve eligible datasets from GEO: (lung OR pulmonary OR LC OR LUAD) AND (miRNA OR microRNA OR miR OR "non-coding RNA" OR ncRNA OR lncRNA OR "noncoding RNA" OR "non coding RNA"). The inclusion criteria were as follows: (i) available expression data of NEAT1 and miR-193a-3p; (ii) a sample size of each dataset in cancer and non-cancerous groups exceeding 3; (iii) human species; and (iv) expression data in the miRNA-related dataset for mature miR-193a-3p.

The following characteristics of each included dataset were collected: first author and published year, region, data source, platform, sample size, and expression level of NEAT1 or miR-193a-3p.

The pooled standard mean difference (SMD) and corresponding 95% confidence intervals (CIs) were calculated to assess the overall expression levels of NEAT1 and miR-193a-3p in LUAD. A summary receiver operating characteristic (sROC) curve was generated to illustrate the ability of NEAT1 and miR-193a-3p expression to discriminate LUAD patients from normal controls. The heterogeneity among included studies was evaluated by the $\chi2$-based Q test and I^2 statistic. A random-effects model was used if there was heterogeneity ($I^2 > 50\%$ or $P < 0.05$). Otherwise, a fixed-effects model would be chosen [22]. Sensitivity analysis was implemented to assess the stability of the pooled SMD. Begg's funnel plot and Egger's test were used to ascertain the possible publication bias. $P > 0.05$ favored no publication bias.

Identification of potential targets of miR-193a-3p based on bioinformatics analysis

Potential targets of miR-193a-3p were forecasted in miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/); this database contains 12 prediction algorithms (TargetScan, RNAhybrid, RNA22, PITA, Pictar2, miRWalk, Microt4, miRNAMap, miRDB, miRBridge, miRanda, and miRmap). Genes appearing in at least 8 algorithms were considered to be putative targets of miR-193a-3p. Additionally, genes positively correlated with NEAT1 were obtained from TCGA. The intersection between the predicted targets and positively correlated genes was determined. Then, we evaluated the expression levels of the overlapping genes with data from TCGA.
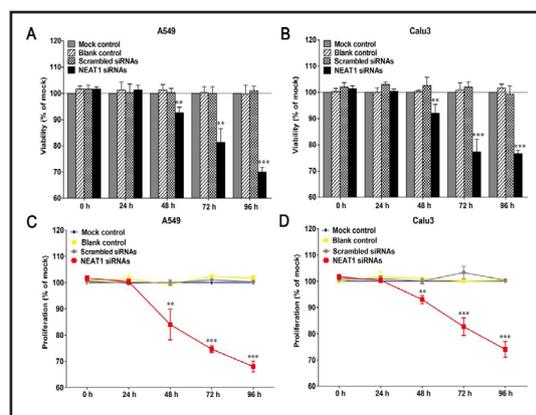
Results

Knockdown of NEAT1 inhibits LUAD cell proliferation and triggers cell apoptosis in vitro

To determine the biological role of NEAT1 in LUAD cell proliferation and apoptosis, we established NEAT1-knockdown cell lines (A549 and Calu3) using siRNAs, with knockdown efficiencies from 75% to 92%. Fluorometric detection of resorufin and an MTS assay showed that silencing of NEAT1 *in vitro* markedly arrested cell viability and proliferation in both A549 and Calu3 cells (Fig. 1). In addition, the effects of NEAT1 on LUAD cell apoptosis were detected by Hoechst 33342/PI double-fluorescent chromatin staining and caspase-3/7 assay. The results indicated that the apoptosis and caspase-3/7 activity of A549 and Calu3 cell lines following NEAT1 knockdown were clearly increased (Fig. 2A-2D). Simultaneously, we observed viable and apoptotic cells under a microscope after Hoechst 33342/PI staining at 96 hours. The results suggested that NEAT1 siRNAs apparently stimulated apoptosis and restrained viability of A549 and Calu3 cell lines (Fig. 2E).

Knockdown of NEAT1 reduces LUAD cell invasion and migration in vitro

Next, we silenced NEAT1 in H1299 cells and subsequently examined the effect of NEAT1 on cell invasion and migration by wound-healing and Transwell assays. Our results showed that the invasion and metastasis of NEAT1-knockdown cells were markedly decreased compared with control cells (Figs. 3, 4), suggesting that NEAT1 siRNAs impeded LUAD cell invasive and metastatic ability *in vitro*.

Fig. 1. NEAT1 siRNAs restrain the viability and proliferation of A549 and Calu3 cells. Mock control, blank control, scrambled siRNAs, and NEAT1 siRNAs were transfected into A549 and Calu3 cells for 0, 24, 48, 72, and 96 hours. (A) Effects of NEAT1 siRNAs on A549 cell viability evaluated by CellTiter-Blue Cell Viability Assay. (B) Effects of NEAT1 siRNAs on Calu3 cell viability evaluated by CellTiter-Blue Cell Viability Assay. (C) Effects of NEAT1 siRNAs on A549 cell proliferation evaluated by MTS assay. (D) Effects of NEAT1 siRNAs on Calu3 cell proliferation by evaluated by MTS assay. *P<0.05; **P<0.01; ***P<0.001.

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:905-918

DOI: 10.1159/000491958
Published online: July 23, 2018

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www.karger.com/cpb

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NEAT1 directly binds to miR-193a-3p in LUAD cells

Multiple studies have demonstrated that lncRNAs could serve as ceRNAs or molecular sponges of miRNAs in multiple malignancies. To determine whether NEAT1 exerts a similar mechanism in LUAD cells, we screened miRNAs containing putative binding sites in NEAT1 with microRNA.org (http://34.236.212.39/microrna/home.do). miR-193a-3p was found to harbor multiple binding sites in NEAT1 (Fig. 5A). Then, we examined the expression of NEAT1 and miR-193a-3p in LUAD cell lines (A549, H1299, and HCC827) and a normal
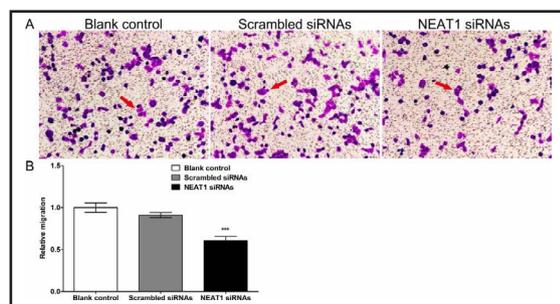
Fig. 2. NEAT1 siRNAs induce apoptosis in A549 and Calu3 cells. (A) Effects of NEAT1 siRNAs on A549 cell apoptosis evaluated by fluorescence microscopy after Hoechst 33342/PI staining. (B) Effects of NEAT1 siRNAs on Calu3 cell apoptosis evaluated by fluorescence microscopy after Hoechst 33342/PI staining. (C) Effects of NEAT1 siRNAs on A549 cell apoptosis per caspase 3/7 activity evaluated by the Apo-ONE® Homogeneous Caspase-3/7 Assay. (D) Effects of NEAT1 siRNAs on Calu3 cell apoptosis per caspase 3/7 activity evaluated by the Apo-ONE® Homogeneous Caspase-3/7 Assay. *P<0.05; **P<0.01; ***P<0.001. (E) NEAT1 siRNAs inhibit the viability and stimulated apoptosis of A549 and Calu3 cells. Hoechst 33342-positive/PI-negative (white arrow): viable cells; Hoechst 33342-positive/PI-negative with blue fragmentation in the cells (red arrow): early apoptotic cells; Hoechst 33342-positive/PI-positive with red fragmentation in the cells (pink arrow): late apoptotic cells; PI-positive with debris signals (yellow arrow): necrotic cells.
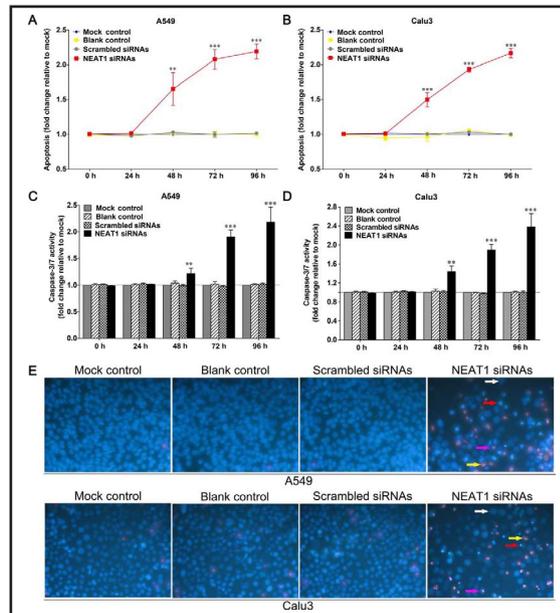

Fig. 3. NEAT1 siRNAs inhibit the migration of H1299 cells. H1299 cells were transfected with blank control, scrambled siRNAs, and NEAT1 siRNAs for 0, 12, 24, and 48 hours. (A) Cell migration was examined in wound-healing assays. (B) The wound width was calculated. The cell migration and would width are shown by red arrows.
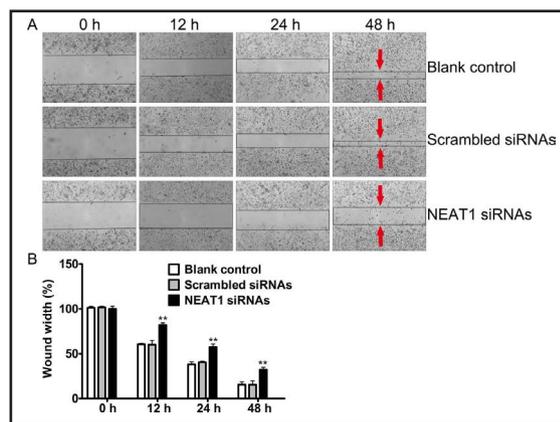
Fig. 4. NEAT1 siRNAs inhibit the invasion of H1299 cells. H1299 cells were transfected with a blank control, scrambled siRNA, and NEAT1 siRNA for 48 hours. (A) Cell invasion was examined with Transwell assays. (B) The number of invasive cells was counted. The invasive cells are shown by red arrows.

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:905-918

DOI: 10.1159/000491958
Published online: July 23, 2018

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Fig. 5. Relationship between NEAT1 and miR-193a-3p. (A) Predicted miR-193a-3p binding sites in NEAT1. (B) Expression levels of NEAT and miR-193a-3p in lung adenocarcinoma (LUAD) cell lines (A459, H1299, and HCC87) and a normal lung epithelial cell line (BEAS-2B). (C) Dual-luciferase reporter assay confirmed that NEAT1 directly bound to miR-193a-3p in LUAD cells.
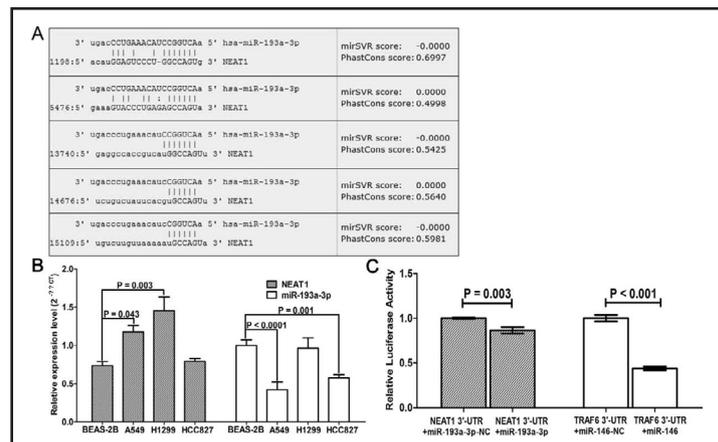
epithelial cell line (BEAS-2B). As shown in Fig. 5B, NEAT1 was significantly upregulated in A549 and H1299 LUAD cells compared with BEAS-2B cells. In addition, miR-193a-3p was clearly downregulated in A549 and HCC827 LUAD cells compared with BEAS-2B cells. Furthermore, to determine whether NEAT1 directly binds to miR-193a-3p, we carried out a dual-luciferase reporter assay. Our results demonstrated that miR-193a-3p decreased the luciferase activity of the NEAT1 3'-UTR, indicating direct binding between NEAT1 and miR-193a-3p (Fig. 5C).

NEAT1 is upregulated while miR-193a-3p is downregulated in LUAD tissues based on meta-analysis

To further elucidate the expression of and correlation between NEAT1 and miR-193a-3p in LUAD, we obtained data from GEO, Oncomine, TCGA, and RT-qPCR on-file to conduct comprehensive analyses. A total of 11 records with 1405 LUAD samples and 415 non-cancerous samples were included for NEAT1 and 10 records with 402 LUAD samples and 213 non-cancerous samples for miR-193a-3p. The basic information on these studies is included in Table 1 and Table 2. The expression of NEAT1 and miR-193a-3p in each dataset is presented as a box-scatter plot and receiver operating characteristic (ROC) curve (Figs. 6–9). Due to the divergences in individual studies, we then combined all of the included datasets. The pooled results showed that the expression level of NEAT1 was higher in LUAD tissues than in normal lung tissues (SMD=0.44, 95% CI=0.32–

Table 1. Main information on the 11 included datasets for NEAT1. T: tumor; N: normal; SD: standard deviation; TCGA: The Cancer Genome Atlas; RT-qPCR: reverse transcription quantitative PCR

| First author (year)	Region	Data source	Platform	Sample size (T/N)	NEAT expression (mean±SD)	
					T	N
Su LJ (2007)	China	GSE7670	GPL96	27/28	8.26±0.87	7.82±0.86
Landi MT (2008)	USA	GSE10072	GPL96	58/49	7.03±0.71	6.84±0.78
Hou J (2010)	Netherlands	GSE19188	GP570	43/63	1.44±1.01	1.24±0.79
Kohno T (2011)	Japan	GSE31210	GPL570	226/20	6.29±0.49	5.96±0.30
Li-Jen S (2013)	China	GSE27262	GPL570	25/25	1.10±0.29	0.92±0.08
Feng L (2014)	China	GSE43767	GPL6480	69/34	11.59±1.47	9.96±2.04
Garber ME (2001)	USA	Oncomine	None	30/6	2.33±1.14	1.32±0.53
Beer DG (2002)	USA	Oncomine	None	86/10	0.23±0.31	0.08±0.07
Okayama H (2012)	Japan	Oncomine	None	226/20	2.40±0.96	2.20±0.51
TCGA (2017)	USA	TCGA	None	535/59	14.55±1.58	13.84±1.20
Pan LJ (2015)	China	In-house RT-qPCR	None	101/101	6.79±3.64	4.92±3.12

Table 2. Main information on the 10 included datasets for miR-193a-3p. T: tumor; N: normal; SD: standard deviation; RT-qPCR: reverse transcription quantitative PCR. T: tumor; N: normal; SD: standard deviation; RT-qPCR: reverse transcription quantitative PCR

First author (year)	Region	Data source	Platform	Sample size (T/N)	MiR-193a-3p expression (mean±SD)	
					T	N
Guled M (2011)	Finland	GSE25508	GPL7731	5/5	6.72±0.75	6.43±0.50
Ma L (2012)	China	GSE29248	GPL8179	3/3	9.73±0.37	9.33±0.32
Ohba T (2013)	Japan	GSE19945	GPL9948	4/8	2.78±1.07	3.59±0.55
van Jaarsveld MT (2013)	Netherlands	GSE47525	GPL17222	6/8	4.02±0.44	3.89±0.97
Bjaanaes MM (2014)	Norway	GSE48414	GPL16770	153/19	1.09±0.60	1.13±0.22
Arima C (2014)	Japan	GSE51853	GPL7341	76/5	0.62±0.48	0.95±0.17
Robles AI (2015)	USA	GSE63805	GPL18410	16/14	0.98±0.92	1.91±1.27
Jin Y (2015)	China	GSE74190	GPL19622	36/44	2.72±1.21	3.14±0.93
Yoshimoto T (2016)	Japan	GSE77380	GPL16170	3/6	4.97±0.67	5.94±0.64
Ren FH (2015)	China	In-house RT-qPCR	None	101/101	4.47±2.82	7.19±2.97

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:905-918

DOI: 10.1159/000491958
Published online: July 23, 2018

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Fig. 6. Box-scatter plots for NEAT1 expression in LUAD. (A) GES7670. (B) GSE10072. (C) GSE19188. (D) GSE31210. (E) GSE27262. (F) GSE43767. (G) Oncomine: Garber ME. (H) Oncomine: Beer DG. (I) Oncomine: Okayama H. (J) TCGA. (K) In-house RT-qPCR.
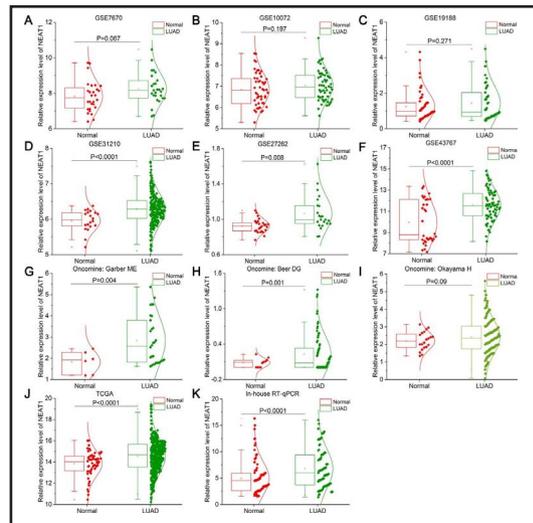
Fig. 7. ROC curves for NEAT1 expression in LUAD. (A) GES7670. (B) GSE10072. (C) GSE19188. (D) GSE31210. (E) GSE27262. (F) GSE43767. (G) Oncomine: Garber ME. (H) Oncomine: Beer DG. (I) Oncomine: Okayama H. (J) TCGA. (K) In-house RT-qPCR.
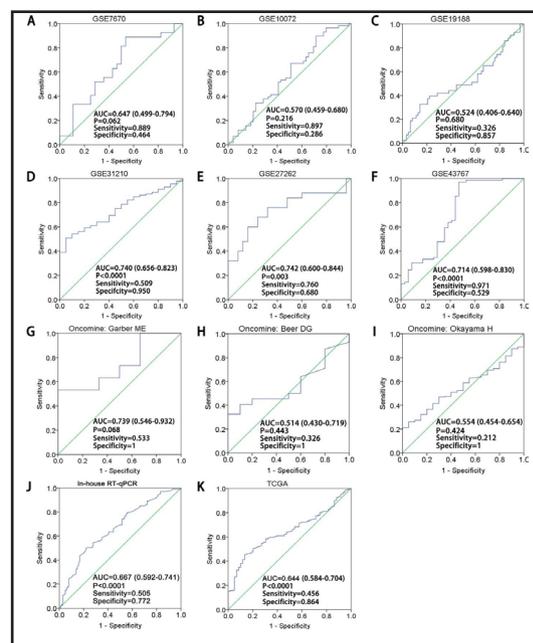

Fig. 8. Box-scatter plots for miR-193a-3p expression in LUAD. (A) GES25508. (B) GSE29248. (C) GSE19945. (D) GSE47525. (E) GSE48414. (F) GSE51853. (G) GSE63805. (H) GSE74190. (I) GSE77380. (J) In-house RT-qPCR.

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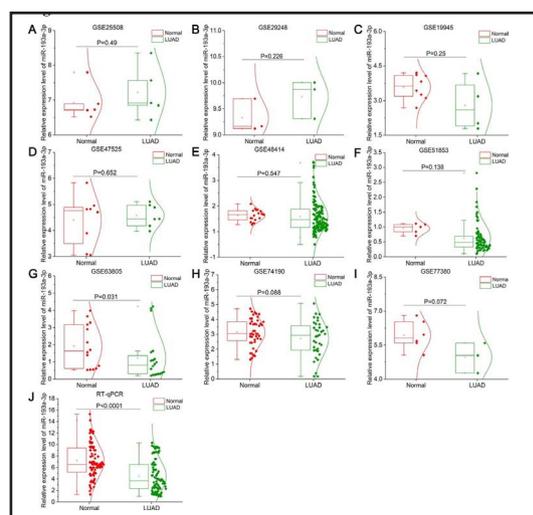
Fig. 9. ROC curves for miR-193a-3p expression in LUAD. (A) GES25508. (B) GSE29248. (C) GSE19945. (D) GSE47525. (E) GSE48414. (F) GSE51853. (G) GSE63805. (H) GSE74190. (I) GSE77380. (J) In-house RT-qPCR.
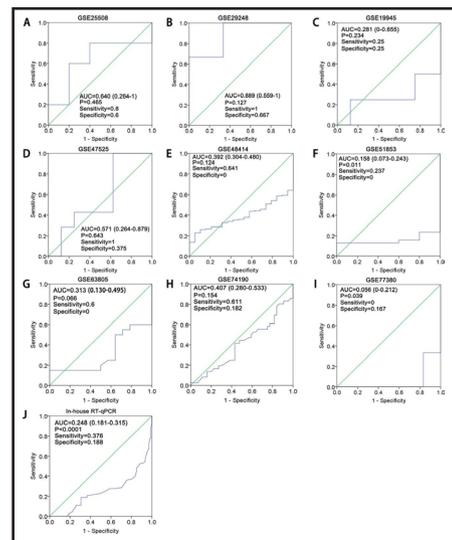
Fig. 10. Expression levels of NEAT1 and miR-193a-3p in LUAD based on data from GEO, Oncomine, TCGA, and our in-house RT-qPCR. (A) Forest plot of 11 records evaluating NEAT1 expression in LUAD (fixed-effects model). If the SMD>0 and the corresponding 95% CI of the SMD did not overlap zero, NEAT1 was upregulated in LUAD tissues compared with normal lung tissues. (B) Forest plot of 10 records evaluating miR-193a-3p expression in LUAD (random-effects model). If the SMD<0 and the corresponding 95% CI of the SMD did not overlap zero, miR-193a-3p was downregulated in LUAD tissues compared with normal lung tissues.
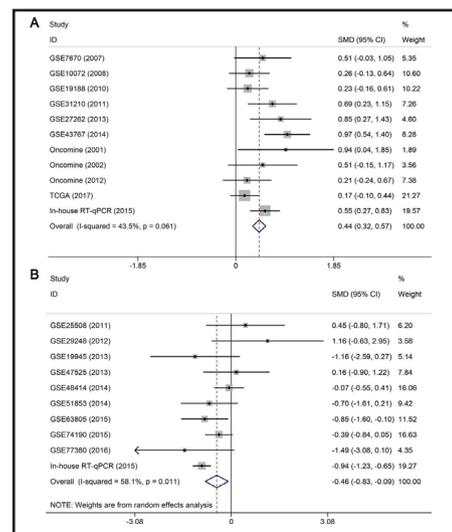
Fig. 11. Sensitivity analysis evaluating meta-analysis stability. (A) Sensitivity analysis indicated that the pooled SMD for NEAT1 was stable. (B) Sensitivity analysis indicated that the pooled SMD for miR-193a-3p was stable.

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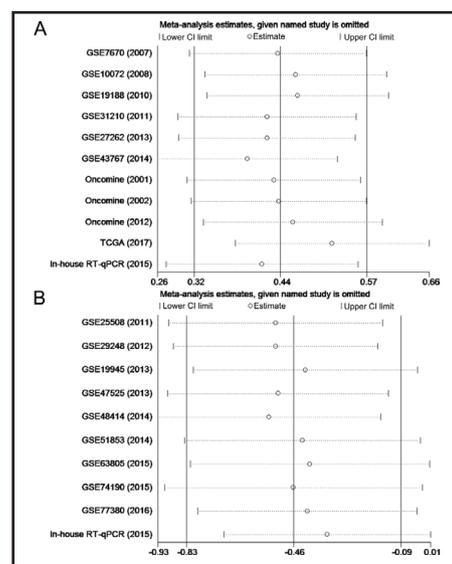
0.57, P<0.0001; I^2=43.5%, P=0.061; Fig. 10A). In contrast, the expression of miR-193a-3p was lower in LUAD tissues than in normal lung tissues (SMD=−0.46, 95% CI=−0.83 to −0.09, P=0.014; I^2=58.1%, P=0.011; Fig. 10B). Sensitivity analyses suggested that the pooled results were stable (Fig. 11). Begg's and Egger's tests indicated no publication bias (NEAT1: Begg's P=0.213, Egger's P=0.137; Fig. 12A; miR-193a-3p: Begg's P=0.721, Egger's P=0.281; Fig. 12B).

Simultaneously, sROC curves were also generated to illustrate the ability of NEAT1 and miR-193a-3p expressions to differentiate LUAD patients from normal controls. As shown in Fig. 13, the area under the curve (AUC) for NEAT1 was 0.75 (95% CI=0.71–0.78), with a sensitivity and specificity of 0.90 (95% CI=0.76–0.96) and 0.43 (95% CI=0.25–0.63), respectively. As for miR-193a-3p, the AUC value was 0.77 (95% CI=0.73–0.80), with a sensitivity and specificity of 0.85 (95% CI=0.61–0.95) and 0.61 (95% CI=0.42–0.76), respectively.

NEAT1 may compete with USF1 for binding to miR-193a-3p as a ceRNA

Putative targets of miR-193a-3p were obtained from 12 prediction algorithms. A total of 285 genes that appeared in at least 8 algorithms were considered targets of miR-193a-3p. Simultaneously, we collected 552 genes positively correlated with NEAT1 from cBioPortal, a database that collects and processes data from TCGA. The intersection between the predicted target genes and positively correlated genes was determined (Fig. 14). In all, six overlapping genes (PIGA, USF1, RGS12, CLEC16A, MMP19, and KHNYN) were obtained for further analyses. The expression patterns of the six genes were visualized in the form of a heatmap and box-scatter plots based on data from TCGA (Fig. 15). According to our results, both USF1 and KHNYN were significantly upregulated in LUAD. We then predicted the binding sites of miR-193a-3p with USF1 and KHNYN and found that only the USF1 gene contained conserved miR-193a-3p binding sites (Fig. 16A). The correlation between USF1 and NEAT1 is shown in Fig. 16B. Furthermore, the overexpression of USF1 in LUAD was confirmed by data from Oncomine (Fig. 17).
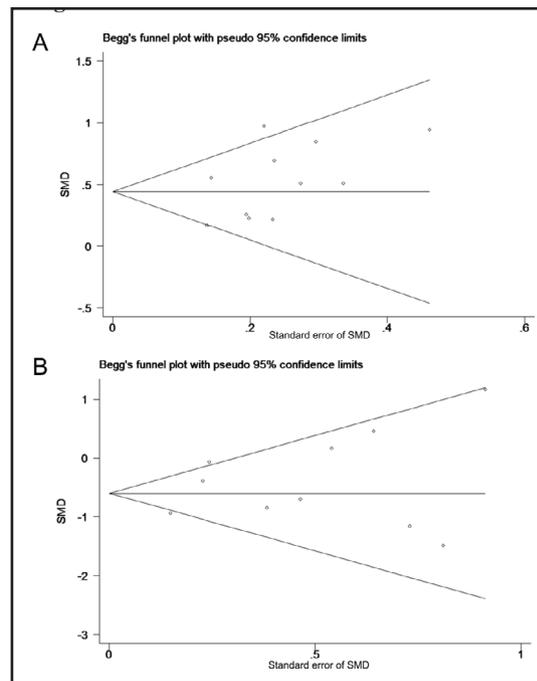

Fig. 12. Potential publication bias among included studies. (A) Funnel plot of 11 included studies for NEAT1. (B) Funnel plot of the 10 included studies for miR-193a-3p.
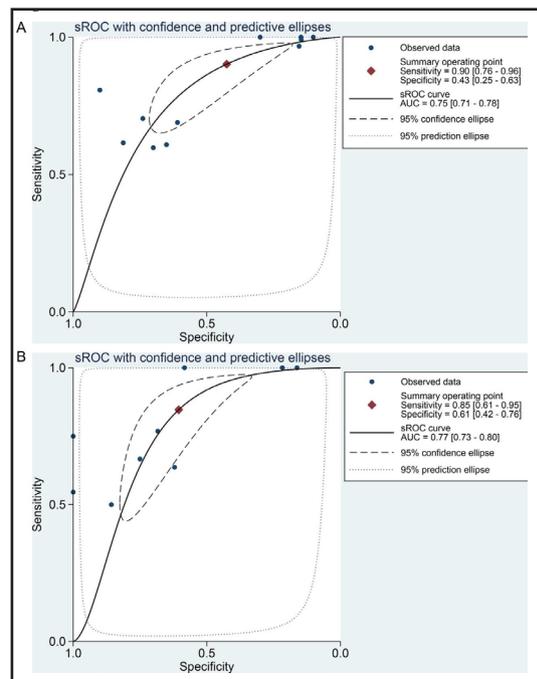

Fig. 13. sROC curves for the identification of LUAD patients from normal controls using NEAT1 and miR-193a-3p expression. (A) Ability of NEAT1 expression to discriminate LUAD patients from normal controls. (B) Ability of miR-193a-3p expression to discriminate LUAD patients from normal controls.

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Fig. 14. Venn diagram of overlapping genes from the predicted target genes of miR-193a-3p and the positively correlated genes of NEAT1.
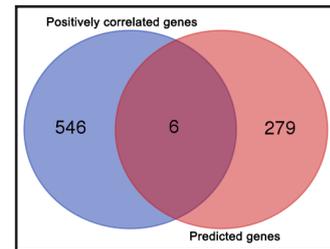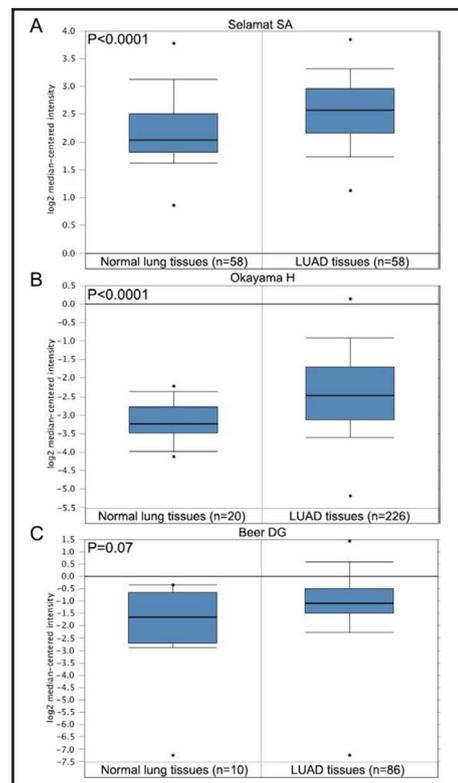

Fig. 15. Expression of the six overlapping genes (PIGA, USF1, RGS12, CLEC16A, MMP19, and KHNYN) in LUAD based on data from TCGA. (A) Heatmap of the six overlapping genes. (B) Box-scatter plots for PIGA, USF1, and RGS12 expression in LUAD. (C) Box-scatter plots for CLEC16A, MMP19, and KHNYN expression in LUAD.
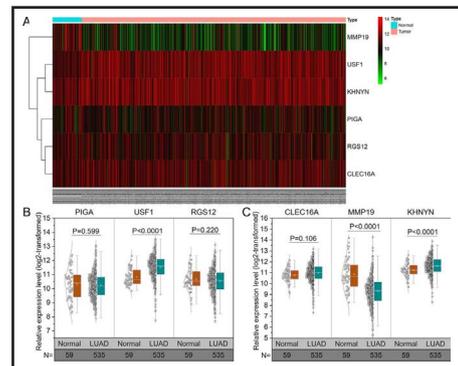
Fig. 16. Identification of USF1 as a potential target of miR-193a-3p. (A) Predicted miR-193a-3p binding sites on USF1. (B) Correlation between USF1 and NEAT1 based on data from TCGA.
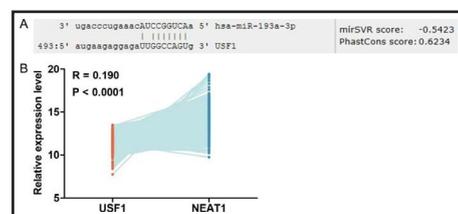
Fig. 17. Expression level of USF1 in LUAD based on data from Oncomine. (A) Study conducted by Semlamat SA. (B) Study conducted by Okayama H. (C) Study conducted by Beer DG.
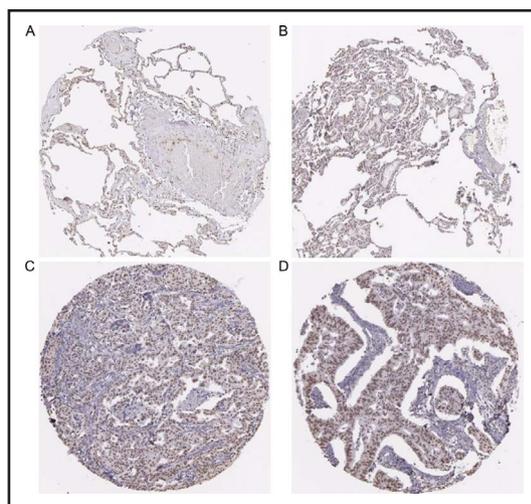
Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:905-918

DOI: 10.1159/000491958
Published online: July 23, 2018

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Because USF1 is a protein-coding gene, we also validated the expression of its encoded protein using data from The Human Protein Atlas database (http://www.proteinatlas.org/). We found that protein USF1 was highly expressed in LUAD tissues compared with normal lung tissues (Fig. 18).

Discussion

Increasing studies have reported that lncRNAs, a class of RNA molecules with more than 200 nucleotides, perform complicated and critical roles in a myriad of malignant tumors at chromatin organizational, translational, and post-translational levels [23-27]. In-depth analysis of the exact actions and mechanisms of specific lncRNAs in tumorigenesis contributes to clinical treatment decision-making in patients with cancers.

The lncRNA NEAT1, an important structural component of paraspeckle, regulates gene transcription and translation

Fig. 18. Immunohistochemical staining of USF1 protein based on The Human Protein Atlas database (https://www.proteinatlas.org/). Paracancerous lung tissues showed moderate USF1 protein expression (A and B). LUAD tissues showed high USF1 protein expression (C and D) Magnification: ×100.

by retaining and editing mRNA in the cell nucleus [28-30]. Multiple reports have indicated that NEAT1 is involved in several malignant tumors, such as hepatocellular carcinoma [31], ovarian carcinoma [32], breast cancer [33], and endometrial carcinoma [34]. The close correlation between NEAT1 expression and NSCLC has also been investigated [11-14].

In this study, we aimed to uncover the biological function and underlying regulatory mechanism of NEAT1 in LUAD. First, we carried out *in vitro* experiments and found that knockdown of NEAT1 in LUAD cells markedly repressed cell proliferation, invasion, and migration and induced cell apoptosis, indicating that NEAT1 could drive LUAD development. However, one limitation should not be ignored in this study: different cell lines were used in different experiments, which may induce inhomogeneity in the results. Then, we focused on the potential molecular mechanism of NEAT1 in LUAD. Previous studies have demonstrated that NEAT1 promoted tumor development by functioning as a ceRNA or a molecular sponge to bind to miRNA and subsequently regulate cancer-related gene expression [35-38]. In this study, we probed the interaction between NEAT1 and miR-193a-3p. Bioinformatics analysis showed that NEAT1 harbored conserved miR-193a-3p cognate sites. Dual-luciferase reporter assay verified that miR-193a-3p directly targeted NEAT1 at its 3'-UTR. We further examined the expression of NEAT1 and miR-193a-3p in LUAD cells and normal lung epithelial cells. The results showed that NEAT1 was upregulated in LUAD cell lines (A549 and H1299) and miR-193a-3p was downregulated in LUAD cell lines (A549 and HCC827) compared with the normal lung cell line (BEAS-2B). However, the expression of NEAT1 in HCC827 cells was similar to that in BEAS-2B cells, and the expression of miR-193a-3p in H1299 cells was similar to that in BEAS-2B cells. Given the different genetic backgrounds of different cell lines, we conjecture that NEAT1 and miR-193a-3p are selectively expressed in different LUAD cell lines. Further study is necessary to corroborate these findings. Our research group has previously detected NEAT1 and miR-193a-3p expression in 125 NSCLC and adjacent non-tumorous lung samples by RT-qPCR, and we found high expression of NEAT1 and low expression of miR-193a-3p in NSCLC tissues [9, 39]. However, the expression levels of NEAT1 and miR-193a in LUAD tissues have not yet been separately elucidated. In this study, we combined the microarray and RNA-seq data from GEO, Oncomine, and TCGA and the in-

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:905-918

DOI: 10.1159/000491958
Published online: July 23, 2018

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house RT-qPCR data from our previous studies to systematically investigate the expression levels of NEAT1 and miR-193a-3p in LUAD. The pooled results showed that NEAT1 was upregulated while miR-193a-3p was downregulated in LUAD tissues. All of these findings indicated a targeted regulation of NEAT1 and miR-193a-3p.

Fig. 19. Underlying regulatory mechanism of NEAT1 in LUAD. NEAT1 may exert its role by functioning as a ceRNA to sponge miR-193a-3p and relieve the inhibitory effect of miR-193a-3p on the USF1 gene.

Numerous studies have demonstrated that miR-193a-3p plays vital roles in various cancers, including lung cancer. Yu et al [40]. detected miR-193a-3p expression in NSCLC and adjacent non-tumorous lung tissues. They found that miR-193a-3p was downregulated in NSCLC tissues and that decreased miR-193a-3p was correlated with TNM stage as well as lymphatic metastasis. The authors also discovered that an enforced expression of miR-193a-3p restrained NSCLC metastasis *in vitro* and *in vivo* by negatively regulating the ERBB4/S6K2 signaling cascade. Similarly, Liang et al [41]. also demonstrated the target effect of miR-193a-3p on ERBB4 in NSCLC. A study carried out by Fan et al [42]. showed that miR-193a-3p has anti-tumor functions via negative modulation of its target KRAS. The interaction between miR-193a-3p and lncRNA has also been reported. A study conducted by Nie et al [43]. indicated that lncRNA UCA1 competitively sponged miR-193a-3p in NSCLC and thus increased the expression of ERBB4, a target of miR-193a-3p. In our study, we demonstrated that NEAT1 could directly bind to miR-193a-3p. To further explore whether NEAT1 acts as a ceRNA and antagonizes the inhibitory effect of miR-193a-3p on its target genes, we searched for putative target genes of miR-193a-3p in 12 online prediction databases and determined genes positively correlated with NEAT1 as candidate targets. Subsequently, we analyzed the expression of these selected genes with data from TCGA. The results showed that the USF1 gene was not only upregulated in LUAD, but also positively correlated with NEAT1. Hence, we hypothesized that NEAT1 may function as a ceRNA to sponge miR-193a-3p and relieve the inhibitory effect of miR-193a-3p on the USF1 gene. The potential regulatory mechanism is shown in Fig. 19. Further *in vitro* and *in vivo* experiments are indispensable to validate our findings.

In summary, our results indicate that an overexpressed NEAT1 facilitates the occurrence and progression of LUAD. NEAT1 may exert its role by functioning as a ceRNA to regulate miR-193a-3p. These findings provide a new perspective on the underlying molecular mechanism of NEAT1 in LUAD, suggesting that NEAT1 could be a valuable biomarker and therapeutic target in patients with LUAD.

Acknowledgements

Disclosure Statement

The authors declare to have no conflict of interests.
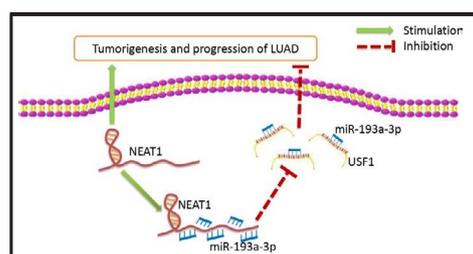
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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:905-918

DOI: 10.1159/000491958
Published online: July 23, 2018

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