Overexpression of HIF-2α-Dependent NEAT1 Promotes the Progression of Non-Small Cell Lung Cancer through miR-101-3p/SOX9/Wnt/β-Catenin Signal Pathway

Xiangjun Kong a, Yue Zhao b, Xinmeng Li a, Zhenxia Tao a, Mingming Hou c, Hui Ma d

aCentral Laboratory, Cangzhou Central Hospital, Cangzhou, China, bRadiotherapy Department, Cangzhou Central Hospital, Cangzhou, China, cMedical Record Management Room, Cangzhou Central Hospital, Cangzhou, China, dDepartment of Medical Safety, Cangzhou Central Hospital, Cangzhou, China

Key Words
NEAT1 • Hypoxia • miR-101-3p • SOX9 • Wnt/β-catenin • NSCLC

Abstract
Background/Aims: The present study aimed to explore the function of NEAT1 on non-small cell lung cancer (NSCLC), as well as its underlying mechanisms. Methods: Quantitative real-time PCR (qRT-PCR) was used to measure NEAT1 expression in NSCLC tissues and cells. MTT assay and transwell assay were performed to detect cell proliferation, migration and invasion. Potential target genes were identified via luciferase reporter assay. Protein analysis was performed through western blotting. Results: The expressions of NEAT1 were significantly higher in both of NSCLC tissues and cells than in normal controls. High expression of NEAT1 was significantly associated with TNM stage ($P=0.000$) and metastasis ($P=0.000$). NEAT1 knockdown inhibited the proliferation, migration and invasion of NSCLC cells. Hypoxia induction mediated by HIF-2α promoted EMT and NEAT1 expressions. Moreover, miR-101-3p was a target of NEAT1. We also found that SOX9 was a target of miR-101-3p. Oncogenic function of NEAT1 on NSCLC progression was mediated by miR-101-3p/SOX9/Wnt/β-catenin signaling pathway. Conclusion: NEAT1 up-regulation induced by HIF-2α over-expression could promote the progression of NSCLC under hypoxic condition. Moreover, NEAT1 also takes part in NSCLC progression via miR-101-3p/SOX9/Wnt/β-catenin axis.

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X. Kong and Y. Zhao contributed equally to this work.

Xiangjun Kong
Central Laboratory, Cangzhou Central Hospital
Cangzhou 061000, Hebei (China)
E-Mail cvxtyrtrt@126.com
Introduction

Lung cancer is a leading cause of cancer-related deaths in the world [1]. Non-small cell lung cancer (NSCLC) is the main sub-type of lung cancer, accounting for 70-80% of all lung cancer cases [2]. Despite great advancements in diagnosis and treatment, NSCLC still sees unfavorable overall survival rate because of tumor metastasis and relapse [3, 4]. Therefore, it is necessary to understand the mechanisms of NSCLC progression and accordingly find more effective therapy methods.

Growing evidences have demonstrated that long non-coding RNAs (lncRNAs) play important roles in various biological processes, including cell proliferation, metastasis and differentiation [5-7]. A number of lncRNAs have been confirmed to be associated with NSCLC, including lncRNA PTV1, MEG3, and HOTAIR [8-10]. However, few studies have focused on the role of nuclear enriched abundant transcript 1 (NEAT1) in NSCLC. NEAT1, located on chromosome 11, derives from the familial tumor syndrome multiple endocrine neoplasia (MEN) type 1 [11] and contains two variants, NEAT1_1 with a length of 3.7kb and NEAT1_2 with a length of 23kb [12]. Abnormal expression of NEAT1 has been observed in several human cancers, like nasopharyngeal carcinoma (NPC) and hepatocellular carcinoma (HCC), suggesting its functions on tumorigenesis [13, 14]. In the present study, we investigated potential role of NEAT1 in NSCLC and possible mechanisms.

Due to aggressive cell proliferation, cancer microenvironment is hypoxic. Previous studies have demonstrated that specific lncRNAs in hypoxia play important roles in cancers [15-17]. Hypoxia-inducible factor (HIF) is involved in major mechanism mediating oxygen-dependent transcriptional responses. HIF-2α, a HIF-α-controlled subunit, regulates cell proliferation, apoptosis, and tumor metabolism under hypoxia [18]. Several published studies have reported that elevated expression of HIF-2α due to hypoxia contributed to the activation of NEAT1 [19]. Therefore, we hypothesized that NEAT1 might boost the development of NSCLC in an HIF-2α-dependent manner under hypoxia.

Wnt/β-catenin signaling pathway plays an important role in regulating the development of multiple tumors, including NSCLC. NEAT1 has been found to serve as a crucial regulator of Wnt/β-catenin [20]. In the present work, we further explored whether NEAT1 could regulate NSCLC progression through Wnt/β-catenin signaling pathway.

Materials and Methods

Tissue sample collection

The study was approved by the Research Ethics Committee of Cangzhou Central Hospital. Written informed consents were signed by all patients in advance.

A total of 131 pairs of NSCLC tissues and adjacent non-tumor ones were obtained from NSCLC patients who received surgery at Cangzhou Central Hospital between 2015 and 2017. The cancer diagnosis was implemented through histopathological evaluation. None of the patients had received any anti-tumor treatments before the operation. All tissue samples were immediately frozen in liquid nitrogen, and stored at -80℃ until RNA extraction.

Cell culture

NSCLC cell lines (A549, SPCA1) and human normal lung cell line (BESA-2B) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, GIBCO), 100 U/ml penicillin and 100 μg/ml streptomycin in humidified air containing 5% CO₂ at 37℃.

Cell counting was completed using a hemocytometer chamber (Qiujing XB-K-25; Shanghai Qiujing Biochemical Reagent and Instrument Co., Ltd., Shanghai, China) under optical microscopy (Olympus CX23; Olympus, Tokyo, Japan) at 40× magnification. In brief, cell culture was digested using 0.25% trypsin, and centrifuged to remove medium; then, the cells were resuspended using PBS buffer and diluted to 100 times.
Subsequently, 10 μL diluted cell suspension was added to each chamber of homogeneate, and observed at 40× magnification. Four counts were recorded for a 1×1-mm area where mean number was calculated. Cells were counted according to the manufacturer’s instructions.

**Cell transfection**

The siRNA of NEAT1 (si-NEAT1) and negative control siRNA (si-NC) were obtained from Ribo-bio (Guangzhou, China). Cells were seeded in 96-well Plates for 24 h before the experiment. These recombinant plasmids were transiently transfected into NSCLC cells using TransMessenger Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

miR-101-3p mimics, miR-101-3p inhibitors and si-SOX9, as well as those corresponding controls were purchased from Guangzhou RiboBio Co. Ltd. These recombinant plasmids were transfected into NSCLC cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol.

**RNA extraction and quantitative real-time PCR**

Total RNA was extracted from cells (10⁷ cells) and tissues (100mg) using Trizol reagent (Life Technologies) according to the manufacturer’s instructions. One Step SYBR® PrimeScript® PLUS RT-RNA PCR Kit (TaKaRa Biotechnology) was used for the Real-Time PCR analysis to test the expression levels of NEAT1. The primers were as follows: forward: 5’-CAGTTAGTTTATCAGTTCTCCCATCA-3’; reverse: 5’-GTGGTGTGTCGCA CCTTCCAACCTCT-3’. In the test for miR-101-3p, RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Biotechnology) was used, and the primers were as follows: forward: 5’-UACAGUACUGUGAUAAACUGA-3’ and reverse: 5’-CAGUUAUCACAGUACUGUAU-3’. β-actin was employed as internal control, and the primers were as follows: forward: 5’-CTGGGACGACATGGAGAAAA-3’ and reverse: 5’-AAGGAAGCTGAAAGATGC-3’.

The reaction was carried out in a system of 20μL, containing 2μL RNA sample, 0.8μL specific primers (0.4μL forward primer and 0.4μL reverse primer) (10μM), 10μL 2×One step TB Green RT-PCR Buffer III, 0.4μL Takara Ex Taq HS (5U/μL), 0.4μL PrimeScript RT enzyme Mix II, 0.4μL ROX Reference Dye or Dye II (×50), and 6μL RNase Free H₂O. The mixture without RNA sample served as negative control. The amplification procedures were performed in Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, California, USA), and contained the following steps: reverse transcription, 42℃ 5min and 95℃ 10sec; PCR procedure: 95℃ 5sec and 60℃ 34sec for 40 cycles. Then, the results were analyzed following Dissociation Protocol. The expression levels of the target genes were calculated using (2⁻ΔΔCt) method.

**MTT assay**

MTT assay was used to evaluate cell viability. 48 h after transfection, the cells were seeded into 96-well plates (5,000 cells/well). Then, 20 µl MTT (with a concentration of 5 mg/ml) was daily added (at hour 24, 48 and 72), and the cells were incubated for an additional 4h in a humidified incubator. A total of 200 μl dimethyl sulfoxide (DMSO) was added into dissolve formazan. Optical density (OD) 490 nm values were measured to estimate cell proliferation.

**Transwell assay**

Cell migration and invasion were determined through Transwell assays (8.0 μm pore size, Costar; Shanghai, China). The upper chamber was coated with 200 μL serum-free DMEM, while the lower ones with 600 μL DMEM supplemented with 10% FBS. For cell invasion assay, the membrane of the chamber was pre-coated with 30 mg/cm² Matrigel (BD Biosciences) for 1 h in 37℃ to form a matrix barrier. 1 × 10⁵ cells/well were seeded to the upper chamber, then the chambers were cultured in 5% CO₂ at 37℃ for 24h. The cells in the lower chamber were fixed with methanol for 10 min and stained with crystal violet for 10 min. Subsequently, cells were washed with PBS. Cell counts were obtained from five randomly selected fields at 200 × magnification.

**Luciferase reporter assay**

Bioinformatics analysis performed via TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/home.do) software demonstrated that the 3’-UTR of NEAT1 possessed binding site for miR-101-3p. The 3’-UTR of NEAT1 containing binding site was amplified with PCR, and cloned into a pmirGlo Dual-luciferase miRNA Target Expression Vector (Promega, Madison, USA) (NEAT1-WT, GenePharma). In addition, the dul-luciferase vector containing the mutational binding site of miR-101-
3p (NEAT1-MUT) was also constructed. HEK293 cells were co-transfected with NEAT1-WT or NEAT1-MUT and miR-101-3p mimic when they reached 50-70% confluence. The cells co-transfected with NEAT1-WT or NEAT1-MUT and miR-NC were employed as negative controls. Dual-luciferase reporter assay kit (Promega, Madison, USA) was used to determine luciferase activity 48h after transfection. Cells were harvested for luciferase assay 48 h after transfection, using luciferase assay kit (Promega, Madison, USA), according to the manufacturer’s protocol.

Wild-type and mutated SOX9 3’UTR (SOX9-wt, and SOX9-mut containing a 6-bp mutation in the predicted binding site of miR-101-3p) luciferase reporter gene Renilla psiCHECK2 vector (Promega, Madison, USA) was constructed. After cultured overnight, cells were co-transfected with the indicated vectors, miR-101-3p mimics and miR-101-3p inhibitor, respectively, using Lipofectamine 2000 (Invitrogen). Luciferase assays were performed as above mentioned.

**Hypoxia culture and HIF-2α over-expression**

NSCLC cells were incubated in an *In vivo* Hypoxia Work Station (Ruskinn Technology Ltd) with an atmosphere of either normoxia (21% oxygen) or hypoxia (1% oxygen) for 12, 24, and 48h. To achieve specific over-expression for HIF-2α, cells were transfected in triplicate with pcDNA3.1-HIF-2α described previously [14].

**Western blot analysis**

The expressions of HIF-2α, E-cadherin, ZO-1, N-cadherin, Vimentin, SOX9, β-catenin and c-Myc proteins in NSCLC cells were detected via western blot analysis. Protein samples were extracted from cells using radioimmunoprecipitation assay buffer (RIPA buffer) with 1% phenylmethylsulfonyl fluoride. Then the protein concentration was determined through bicinchoninic acid (BCA) protein quantification. The proteins (20 µg/lane) were separated applying 10% SDS-PAGE minigel, and subsequently transferred onto polyvinylidene fluoride membrane. Membranes were blocked adopting 5% nonfat dry milk in PBS-0.1% Tween for 1 h, and then probed at 4˚C overnight with primary antibodies as follows: anti-HIF-2α, anti-E-cadherin, anti-ZO-1, anti-N-cadherin, anti-Vimentin, anti-SOX9, anti-β-catenin, anti-c-Myc and anti-GAPDH. The blots were subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000, Abcam). Enhanced chemiluminescence substrate was used to visualize the signals (EMD Millipore, Billerica). GAPDH was used as loading control.

**Statistical analysis**

Statistical analysis was accomplished using SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). Continuous data were presented as mean±standard deviation (SD), and compared between two groups using the Student’s t-test. Chi-square test was used to analyze the association of gene expression with clinical features of the patients. *P*<0.05 indicated statistical significance of the results.

**Results**

The up-regulation of NEAT1 was correlated with malignant progression of NSCLC

QRT-PCR was performed to detect the expression of NEAT1 in NSCLC tissues and adjacent normal tissues as well as in lung cancer cell lines A549 and SPC-A1, and human normal lung cell line (BESA-2B). The results showed that NEAT1 expression was higher in NSCLC tissues than in adjacent normal lung tissues (*P*<0.05, Fig. 1A). Moreover, the expression level of NEAT1 in NSCLC cell lines (A549 and SPCA1) were also significantly higher than that in BESA-2B (*P*<0.05, Fig. 1B).

Chi-square test was performed to estimate the association of NEAT1 with clinical characteristics of NSCLC patients. The NSCLC patients were divided into high (n=66) and low (n=65) level groups according to their median expressions of NEAT1 in NSCLC tissue specimens (1.8). Analysis results revealed that NEAT1 expression was positively associated with TNM stage (*P*=0.000) and lymph node metastasis (*P*=0.000) (Table 1).
Knockdown of NEAT1 suppressed NSCLC cell proliferation, migration and invasion

QRT-PCR was used to determine the transfection efficiency of si-NEAT1 in NSCLC cell lines. As shown in Fig. 2A-B, the expression of NEAT1 was significantly down-regulated in A549 and SPCA1 cells after si-NEAT1 transfection (P<0.01). Furthermore, MTT assays revealed that the proliferation of both A549 and SPCA1 cells was inhibited in si-NEAT1 group, compared to the controls (Fig. 2C-D). The migration and invasion assays showed that NEAT1 knockdown significantly suppressed cell migration and invasion (Fig. 2E-F). These data revealed that NEAT1 knockdown inhibited metastatic behaviors of NSCLC cells.
Increased expression of NEAT1 and HIF-2α under hypoxia

The expression of NEAT1 was increased in A549 and SPCA1 cells after hypoxic treatment for 12, 24 and 48h, and reached the highest level at 24h ($P<0.01$, Fig. 3A-B). In addition, western blot showed that the expressions of HIF-2α and EMT-associated proteins (N-cadherin and Vimentin) were increased in A549 and SPCA1 cells after hypoxic treatment for 12, 24 and 48h, especially at 24h. While E-cadherin and ZO-1 expressions were decreased (Fig. 3C-D). Moreover, the migration and invasion of A549 and SPCA1 cells were also increased under hypoxic condition for 12, 24 and 48h, especially at 24h (Fig. 3E-F). All data indicated that NEAT1 expression and EMT activity were obviously enhanced under hypoxic condition.
NEAT1 promoted EMT and NSCLC cell metastasis regulated by HIF-2α

Experiment was performed after 24 h hypoxia and A549 was adopted. To define the influence of HIF-2α on NEAT1 expression, A549 cells were transfected by pcDNA3.1-HIF-2α, and cultured under hypoxic condition for 24h. The results showed that HIF-2α overexpression enhanced the expressions of NEAT1, N-cadherin and Vimentin, and promoted cell migration and invasion under hypoxic condition, compared with untreated cells; whereas, E-cadherin and ZO-1 protein levels were decreased (P<0.05, Fig. 4A-C). These data suggested that NEAT1 promoted EMT and NSCLC cells’ metastasis under hypoxia in a HIF-2α-dependent manner.
miR-101-3p could directly bind to NEAT1

We detected the expression of miR-101-3p in A549 and SPCA1 cells before and after si-NEAT1 transfection. The results showed that si-NEAT1 transfection increased miR-101-3p expression (P<0.05, Fig. 5A-B). However, up-regulated miR-101-3p expression did not influence the expression of NEAT1 (P>0.05, Fig. 5C-D). Thus, miR-101-3p might be a potential target of NEAT1.

Bioinformatic analysis demonstrated that NEAT1 3’UTR possesses binding site for miR-101-3p (Fig. 6A and B). To further investigate whether miR-101-3p is a target of NEAT1, the dual-luciferase reporter assay was performed. Our results showed that the luciferase activity was significantly decreased after co-transfection with miR-101-3p and WT-NEAT1 when compared to co-transfection with miR-NC and WT-NEAT1. Meanwhile, the co-transfection with miR-101-3p and MUT-NEAT1 did not change the luciferase activity, suggesting that miR-101-3p might bind to 3’UTR of NEAT1 (Fig. 6C). All of the data revealed that miR-101-3p might be a down-stream target of NEAT1.

SOX9 was a target gene of miR-101-3p

TargetScan was used to predict target genes of miR-101-3p, and located SOX9 as a candidate gene (Fig. 7A). In order to verify this prediction, the dual-luciferase reporter assay was performed. The results showed that miR-101-3p over-expression significantly reduced the luciferase activity for wt-3’-UTR of SOX9, but not for mut-3’-UTR of SOX9 (Fig. 7B).
Moreover, western blot was performed to further detect targeted relationship between miR-101-3p and SOX9. The results showed that increased expression of miR-101-3p significantly decreased the level of SOX9 protein (Fig. 7C). Therefore, SOX9 was a target gene of miR-101-3p in NSCLC.
miR-101-3p regulated the effect of NEAT1 on SOX9

In order to explore regulatory relationship between NEAT1 and SOX9, we detected the expression of SOX9 in NSCLC cells transfected with si-NEAT1. The data showed that NEAT1 knockdown significantly reduced the expression of SOX9 (Fig. 8A). In addition, we found that si-NEAT1 could reduce SOX9 expression without miR-101-3p inhibitor (Fig. 8B). These findings suggested that NEAT1 controlled SOX9 expression through miR-101-3p.

Fig. 7. SOX9 is a target of miR-101-3p. (A) A schematic illustration of wt and Mut 3’-UTR of SOX9. (B) Dual-luciferase reporter assay revealed that miR-101-3p inhibited wt SOX9 3’-UTR luciferase activity, but not Mut SOX9 3’-UTR luciferase activity. (C) SOX9 expression was determined with western blot in A549 and SPCA1 cells transfected with miR-101-3p mimics or miR-NC. *, P<0.05.

Fig. 8. SOX9 protein expression in A549 and SPCA1 cells measured with Western blot. (A) SOX9 protein levels in cells transfected with si-NEAT1 or si-NC. (B) SOX9 protein expressions in cells transfected with si-NEAT1 or si-NC with or without miR-101-3p inhibitor.
Fig. 9. The effect of miR-101-3p and SOX9 on the Wnt/β-catenin signaling pathway in A549 and SPC-A1 cells. (A) SOX9 activated the Wnt/β-catenin signaling pathway. (B) The protein levels of SOX9, β-catenin and c-Myc in cells transfected with miR-101-3p mimics or miR-101-3p inhibitor.

Discussion

lncRNAs are significantly associated with tumor invasion and metastasis [22]. For instance, Nie et al. reported that lncRNA ANRIL could promote NSCLC cell proliferation and inhibit cell apoptosis via silencing KLF2 and P21 expressions [23]. In the study by Chen et al., lncRNA TUBA4B was found to be able to regulate the proliferation of NSCLC cells, acting as a potential prognostic biomarker for the cancer [24]. However, the role of NEAT1 in NSCLC and possible underlying mechanisms thereof are still unclear.

The functional roles of NEAT1 have been confirmed in various malignancies. For example, Chen et al. found that NEAT1 was related to malignant characters of ESCC, including cell proliferation, migration and invasion, and that NEAT1 over-expression could independently influence patients prognosis [25]. Li et al. reported that NEAT1 promoted endometrioid adenocarcinoma invasion and migration via regulating c-Myc, IFG1, MMP-2 and MMP-7 [26]. Moreover, in the study by Zhen et al., NEAT1 was revealed to promote glioma cell proliferation, invasion and migration via miR-449b-5p/c-Met axis [27]. However, in the study by Wang et al., NEAT1 expression was reduced in nasopharyngeal carcinoma, and NEAT1 inhibited NPC cell growth, invasion and radiation resistance in vitro as well as
tumor metastasis in vivo [13]. The above findings reveal that NEAT1 may serve as a tumor oncogene or suppressor in different tumors. Such opposition might be attributed to different types of cancer tissues. That is, different types of cancer tissues may show significantly diverse sensitivities to the same gene driven by aneuploidy patterns, thus forming tissue-type-specific genetic network [28].

In the present study, we found that NEAT1 functioned as an oncogene in NSCLC. The expression of NEAT1 was higher in both NSCLC tissues and cells than in the normal controls. And increased NEAT1 expression in NSCLC tissues was significantly correlated with aggressive clinicopathological features. NEAT1 up-regulation might predict disease progression, but its effect on the prognosis of NSCLC was not explored in our study due to the relatively small sample size and the lack of follow-up investigation. The experiments showed that NEAT1 knockdown inhibited NSCLC cells proliferation, migration and invasion in vitro. Moreover, we detected the function of NEAT1 under hypoxic condition as well, considering the fact that tumor growth could cause hypoxia owing to rapidly growing tumor mass outstripping its vasculature and the lack of oxygen and nutrients [29]. Our results showed that HIF-2α and NEAT1 levels were up-regulated under hypoxic condition. Moreover, increased expression of NEAT1 was induced by HIF-2α over-expression. NEAT1 up-regulation promoted EMT and NSCLC cells’ metastasis under hypoxic condition. However, exact molecular mechanism of NEAT1 prompting EMT and NSCLC metastasis remains unknown.

According to previous studies, lncRNA could act as a miRNA sponge to regulate gene expression, thus taking part in biological processes. For instance, Shao et al. reported that lncRNA RMRP could acted as a miR-206 sponge to modulate gastric cancer cell cycle via regulating Cyclin D2 expression [30]. Li et al. suggested that lncRNA UCA1 promoted glutamine metabolism through targeting miR-16 in human bladder cancer [31]. In this study, miR-101-3p was identified as a potential target of NEAT1 via luciferase reporter assay. The results showed that NEAT1 down-regulation could increase miR-101-3p expression, but that increased miR-101-3p expression had nothing to do with NEAT1 expression. Thus, miR-101-3p was a direct target of NEAT1.

To further elucidate potential molecular mechanism underlying oncogenic function of NEAT1 on NSCLC progression, we focused on the association between NEAT1 and Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling is known to regulate a broad range of cellular processes [32]. Recent studies have showed a significant correlation between Wnt/β-catenin signaling pathway and SOX9 in tumor progression. For example, Santos et al. found that SOX9 up-regulation could activate Wnt signaling to participate in gastric cancer progression [33]. Ma et al. reported that SOX9 enhanced Wnt pathway activation in prostate cancer [34]. Prévostel et al. showed that SOX9 was an atypical intestinal tumor suppressor controlling Wnt/β-catenin signaling [35]. In our study, SOX9 could act as a target of miR-101-3p, while NEAT1 played an oncogenic role in NSCLC progression through activating miR-101-3p/SOX9/Wnt/β-catenin axis. However, these results were not verified in vivo due to the short period of our study. Therefore, proper animal experiments are recommended to verify and improve our findings.

**Conclusion**

In conclusion, NEAT1 acts as an oncogenic IncRNA in NSCLC, and promotes NSCLC tumorigenesis and progression via miR-101-3p/SOX9/Wnt/β-catenin axis. Moreover, excessive activation of NEAT1 could contribute to EMT and metastasis in NSCLC in an HIF-2α-dependent way. NEAT1 may be a promising therapeutic target for NSCLC.

**Disclosure Statement**

No competing financial interests or conflicts of interest exist.
References


