European Review for Medical and Pharmacological Sciences 2017; 21: 504-510

Long non-coding RNA NEAT1 acts as oncogene in NSCLC by regulating the Wnt signaling pathway

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Abstract. – OBJECTIVE: The present study aimed to explore the role of long non-coding RNA NEAT1 (NEAT1) in mediating non-small cell lung cancer (NSCLC) cell migration and invasion, as well as the underlying regulatory mechanisms.

PATIENTS AND METHODS: The NEAT1 expression in NSCLC tissues and cell lines was measured using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The relationships between NEAT1 expression and clinicopathological parameters were examined by chi-square test. Overall survival curves were analyzed using the Kaplan-Meier method. Effects of NEAT1 on cell growth, invasion and migration were evaluated by cell counting kit-8 assay and transwell assay, respectively. Western blotting was used to address the impact of NEAT1 on Wnt/β-catenin signaling.

RESULTS: We observed that the expression of NEAT1 in NSCLC tissues and cell lines were much higher than that in normal control, respectively. High NEAT1 expression was statistically associated with poor differentiation, Lymph node metastasis and advanced TMN stage (all $p < 0.05$). According to the Kaplan-Meier survival analysis, NSCLC patients with high NEAT1expression had a significantly shorter overall survival than those with high NEAT1 expression($p < 0.001$). Moreover, the downregulation of NEAT1 expression significantly inhibited the NSCLC cells proliferation, migration, and invasiveness. Finally, we found that decreased expression of NEAT1 inhibited the Wnt/β-catenin signaling pathway activity.

CONCLUSIONS: Our data for the first time showed that NEAT1 contribute to the tumorigenesis and development of NSCLC by activating Wnt/β-catenin signaling pathway, suggesting that NEAT1 may provide a therapeutic strategy for the treatment of NSCLC patients.

Key Words:
Long non-coding RNA, NEAT1, NSCLC, Wnt/β-catenin, Prognosis.

Introduction

Lung cancer is the most frequent cause of cancer-related deaths worldwide[1]. Non-small cell lung cancer (NSCLC) accounts for 70-80% of all lung cancer cases, and nearly 50% of patients with stage I NSCLC die within 10 years after initial diagnosis[2,3]. Although with the development of treatment methods, unfortunately, the five-year survival rate of NSCLC still has no significant increased owing to early tumor metastasis and relapse[4]. It is highly necessary to explore novel therapeutic strategies to limit or reduce the ability of invasion and migration of NSCLC cells.

More and more studies have focused on long non-coding RNAs (lncRNAs), which is a length of more than 200 nucleotides and without protein-coding potential[5]. LncRNAs play important regulatory roles in various ways, such as cell cycle control, DNA methylation, as well as transcription and transcriptional regulation[6]. Recently An increasing number of lncRNAs have been found to play critical roles in cancer development and metastasis, including NSCLC. For example, Wang et al[7] showed that long non-coding RNA TUSC7 promoted NSCLC growth and correlated with prognosis of NSCLC patients. Li et al[8] reported that long non-coding RNA AGAP2-AS1 could promote cell proliferation, migration, and invasion, and induced cell apoptosis through interacting with EZH2 and LSD1 in NSCLC cells. However, there are little studies about the role of nuclear enriched abundant transcript 1 (NEAT1) in NSCLC.

Wnt/β-catenin signaling pathway played a crucial role in regulating multiple aspects of tumor development, including NSCLC. It has been known that Wnt/β-catenin signaling pathway

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plays a critical role in cancer invasion and metastasis. NEAT1 is a novel long non-coding RNA (lncRNA) which served as a crucial regulator in several cancers[9,10]. In the present work, we explored the association between NETA1 expression and clinicopathological significance. Subsequently, we completed a series of *in vitro* experiments to investigate the role of NETA1 in NSCLC proliferation and metastasis. Finally, we explore whether NETA1 could regulate Wnt/β-catenin signaling pathway.

Patients and Methods

Specimens

133 paired NSCLC and adjacent non-tumor specimens were collected from the Department of Oncology, General Hospital of People's Liberation Army. All tissue samples were flash-frozen in liquid nitrogen immediately after collection and stored at -80°C until use. None of the patients had received preoperative adjuvant therapy. For the use of these clinical materials for research purposes, written informed consent was obtained from all the participants. This study was approved by the Ethics Committee of General Hospital of People's Liberation Army.

Cell Culture and Transfection

The human normal the normal lung cell line (BEAS-2B), and the human NSCLC cell lines (A549, H1299, SPCA1 and H358) were obtained from the Cell Bank of Chinese Academy of Science (Songjiang, Shanghai, China). The cells were maintained in F-12K Medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The siRNA of NEAT1 and negative control siRNA (si-NC) were obtained from Ribobio (Guangzhou, China). Cells were seeded in 96- or 6-well Plates 24 h before the experiment. These oligonucleotides were transiently transfected into NSCLC cells using TransMessenger Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative RT-PCR

Total RNA was extracted with TRIzol reagents (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was synthesized by using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Otsu, Shiga, Japan). The quantitative PCR was performed on the cDNA with specific primers for NEAT1. The GAPDH was served as an internal control. QRT-PCR reactions were performed by using an ABI7500 system (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Takara, Otsu, Shiga, Japan). Relative expression of NEAT14 was calculated using the comparative cycle threshold (CT) ($2^{-\Delta\Delta CT}$) method. The primer used in this study was shown as follows, NEAT1: forward 5'-GUCUGUGUGGAAGGAGGAATT-3',

reverse 5'-UUCCUCCUUCCACACAGACTT-3'.

GAPDH: Forward 5'-TGAACGGGAAGCTCACTGG-3',

reverse 5'-TCCACCACCCTGTTGCTGTA-3'.

Cell Proliferation by CKK-8 Analysis

A549 and H1299 cells (5x103/well) were seeded into 96-well plates. Then the CCK-8 reagent was added to culture medium 72h after transfection. Absorbance in each well was measured by using a microplate reader set at 570 nm.

Cell Migration and Invasion Assays

The invasive and migration behaviors of NEAT1 cells were determined by Transwell chamber(Corning Costar Corp., Cambridge, MA, USA). NSCLC cells were cultured in 6-well plate. After approaching almost 100% confl uence, the cells were scratched with a 20 μl tips, followed by washing with PBS and treatment with 0, 15, 20 and 25 μM of PEITC for 24 h. The cell numbers were counted from five nonoverlapping fields of each membrane. Three independent assays were performed.

Western Blot

Collected cells were lysed immediately in RIPA buffer supplemented with a protease inhibitor cocktail (Calbiochem, San Diego, CA, USA). Samples were electrophoresed by using 10% SDS-PAGE. The protein was then transferred onto a PVDF (polyvinylidene fluoride) membrane (Bio-Rad, Beijing, China). After blocking in skim milk, the membranes were incubated with specific antibodies. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad, Beijing, China). All the antibodies were brought from the Bioworld Company (St. Paul, MN, USA). Finally, the membrane was developed by enhanced ECL (Beyotime Biotechnology, Bei-jing, China).

Statistical Analysis

Statistical analyses were performed using SPSS 17.0 computer software (SPSS Inc., Chi-

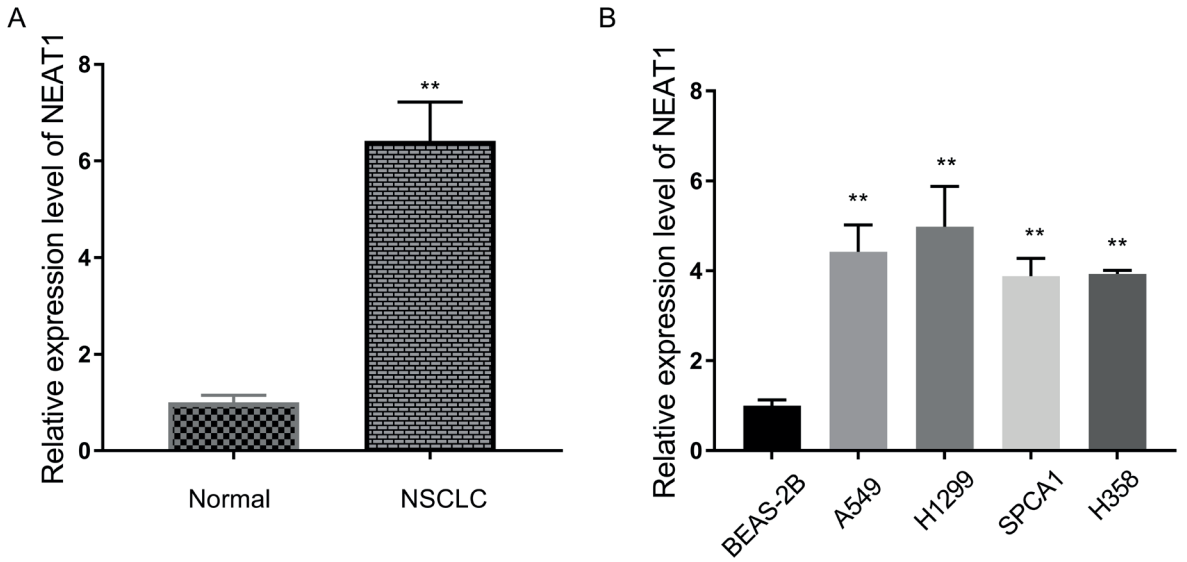

Figure 1. Expression of NEAT1 in NSCLC tissues and cell lines. ***(A)*** Levels of NEAT1 that were detected in NSCLC tissues were significantly higher than the levels of NEAT1 detected in the corresponding adjacent, non-cancerous tissues. ***(B)*** Levels of NEAT1 in NSCLC cell lines (A549, H1299, SPCA1 and H358) were significantly higher than in normal lung cell line (BEAS-2B). $^{**}p < 0.01$.

cago, IL, USA). All the experiments were carried out at least three times, and all samples were analyzed in triplicate. The Student's t-test was used to analyze the differences in NEAT1 expression between the tumor and normal tissues. The relationships between the NEAT1 expression level and clinicopathological parameters were analyzed using the Pearson χ^2 test. Kaplan-Meier analyses were used to analyze overall survival. All differences were statistically significant at the level of $p < 0.05$.

Results

Expression Levels of NEAT1 in NSCLC Tissues and Cell lines

To explore whether NEAT1 was upregulated in NSCLC, we first performed RT-PCR to determine the levels of NEAT1 in NSCLC. Our results showed that the relative level of NEAT1 was significantly higher in NSCLC compared to the adjacent normal lung tissues ($p = 0.001$, Figure 1A). Next, we further explore the expression levels of NEAT1 in four NSCLC cells lines (A549, H1299, SPCA1 and H358). Our data showed that higher level of NEAT1 was observed in the NSCLC cell lines (A549, H1299, SPCA1 and H358) compared with the human normal lung cell line, BEAS-2B ($p < 0.01$, respectively, Figure 1B).

NEAT1 upregulation was Associated with Aggressive Progression in NSCLC and Predicted poor Prognosis in Patients with NSCLC

To explore the clinical significance of NEAT1 in NSCLC patients, associations between NEAT1 expression and various clinicopathologic features of glioma patients were evaluated statistically. We found that high NEAT1 expression was statistically associated with poor differentiation, Lymph node metastasis and advanced TMN stage (all $p < 0.05$, Table I). Furthermore, we use the Kaplan–Meier method and log rank test to detect the overall survival of patients with NSCLC. Our results showed that NSCLC patients with high NEAT1 expression had a significantly shorter overall survival than those with high NEAT1 expression ($p < 0.001$, Figure 2).

Knockdown of NEAT1 Suppressed NSCLC Cell Proliferation, Migration, and Invasion

To validate the transection efficiency, the relative expression levels of NEAT1 in NSCLC cells after transfection of si-NEAT1 were detected by qRT-PCR. As shown in Figure 3A-B, the NEAT1 expression level was successfully downregulated in A549 and H1299 ($p < 0.001$, respectively). Next, CKK-8 assays were carried out to explore the functional role of NEAT1 in NSCLC

Table I. Correlation Between NEAT1 Expression and clinicopathological parameters of NSCLC.

Clinicopathological parameters	No. of cases	Relative NEAT1 expression		*p*
		High	Low	
Age (years)				0.856
≤65	55	34	21	
>65	78	47	31	
Gender				0.146
Male	74	41	33	
Female	59	40	19	
Differentiation				0.005
Well, moderate	67	32	34	
Poor	67	49	18	
Tumor size				0.088
≤5 cm	66	36	31	
>5 cm	67	45	21	
Lymph node metastasis				0.006
Positive	71	51	20	
Negative	62	30	32	
TMN stage				0.001
I	71	33	38	
II/III/IV	62	48	14	

cells by determining the effects of knockdown of NEAT1 in A549 and H1299 cell lines. The results showed that si-NEAT1 effectively inhibited cell viability in both A549 and H1299 (Figure 3C-D). In addition, *in vitro* migration assays and invasion assays were also performed. The results showed that Knockdown of NEAT1 significantly inhibited cell migration (Figure 2E-F, Both $p < 0.001$) and invasion (Figure 3G-H, Both $p < 0.001$). These data demonstrate that NEAT1 knockdown inhibits metastatic behaviors of NSCLC cells both *in vitro*.

NEAT1 Contributed to the Activation of Wnt/β-catenin Signaling in NSCLC Cells

To investigate the possible molecular mechanism of NEAT1 regulating NSCLC cells behavior, we focused on the Wnt/β-catenin signaling pathway, based on Wnt/β-catenin pathway plays a very important role in the initiation and progression of NSCLC. As shown in Figure 4, Western blot showed that decreased expression of NEAT1 leads to significant decreased β-catenin expression in si-NEAT1 transfected NSCLC cells compared with the si-NC group. Moreover, we also found that the transfection of NEAT1 inhibitor significantly suppressed the protein expression levels of cyclin D1 and c-myc. These results indicated that NEAT1 expression may regulate the activity of WNT/β-catenin signaling.

Discussion

Although many studies showed lncRNA expression signatures in a variety of malignant human cancers for decades[11], elucidation of the role of the dysregulation of specific lncRNAs in carcinogenesis remains in the initial stages of development. In the present study, we found that the expression of NEAT1 in NSCLC tissues and cell lines were much higher than that in normal control, respectively, which is consistent with previous studies focused on other human malignancies[12,13]. We also found that increased NEAT1 expression in NSCLC tissues was significantly correlated with aggressive clinicopathological features. Also, based on the

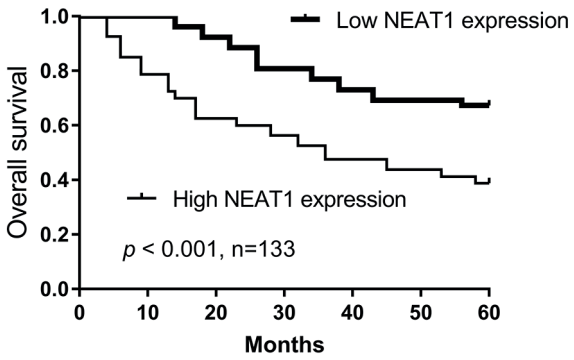

Figure 2. Kaplan-Meier survival curves showed that patients with high NEAT1 expression demonstrated poorer clinical outcome ($p < 0.001$).

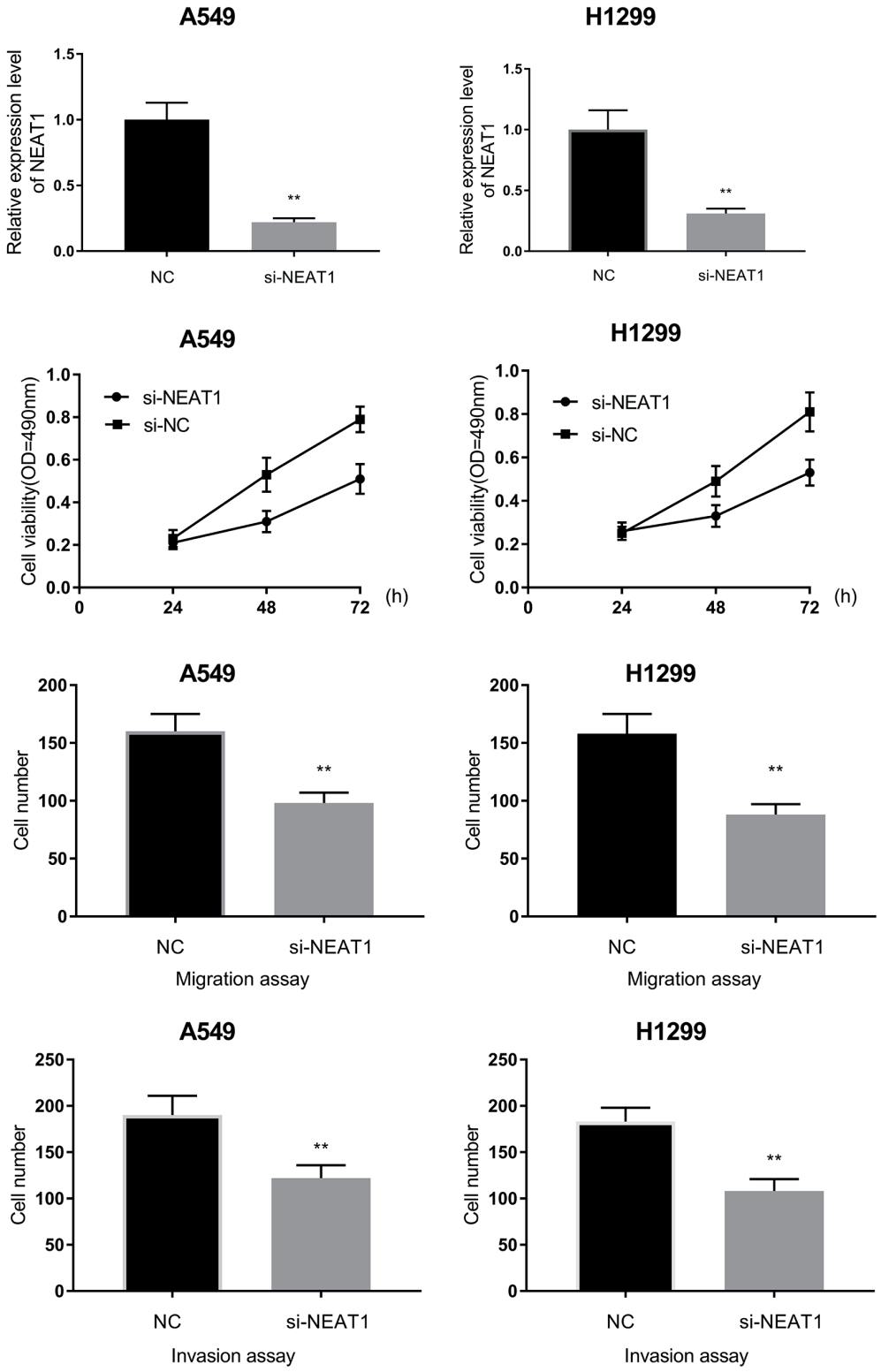

Figure 3. NEAT1 inhibits NSCLC cell proliferation, migration, and invasion. (A,B) NEAT1 knockdown efficiency was confirmed by RT-qPCR in NSCLC cells (A549, H1299). (C,D) CKK-8 analysis of the effects of NEAT1 on the proliferation of A549 and H1299. (E,F) Transwell assay was conducted to analyze the migration of A549 and H1299. (G,H) Transwell assay was conducted to analyze the invasion of A549 and H1299. $^{**}p < 0.01$.

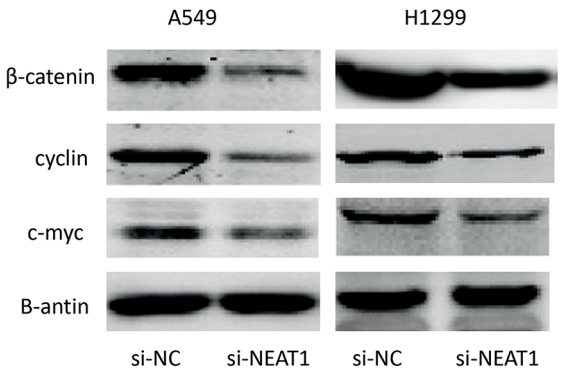

Figure 4. Inhibited NEAT1 expression inhibited the activation of Wnt/β-catenin signaling pathway.

Kaplan-Meier method, we observed that NEAT1 overexpression was associated with lower overall survival rates. Further *in vitro* experiment showed that knockdown of NEAT1 could suppress NSCLC cells proliferation, migration, and invasion. Those results revealed that NEAT1 may play an important role in development, tumorigenesis, and progression of NSCLC.

NEAT1 has been extensively demonstrated to be involved in several cancers. For instance, Chen et al[14] found that NEAT1 contributes to the malignant characters of ESCC through involvement in proliferation, migration, and invasion, and over-expression of NEAT was an independent risk factor of overall survival. Li et al[15] reported that NEAT1 promoted endometrial endometrioid adenocarcinoma invasion and migration via regulating c-myc, IFG1, MMP-2, and MMP-7. Zhen et al showed that NEAT1 promoted glioma cell proliferation, invasion, and migration by regulating miR-449b-5p/c-Met axis[16]. Most recently, Sun et al[17] reported that NEAT1 was highly expressed in patients with NSCLC. Moreover, they identified that NEAT1 could be a crucial oncogenic regulator through acting as a ceRNA for miR-377-3p. Those results revealed that NEAT1 served as a tumor promoter in several tumors, including NSCLC.

To elucidate the molecular mechanism by which NEAT1 promote NSCLC migration and invasion, we focused on the association between NEAT1 and Wnt/β-catenin signaling pathway. The effect of Wnt/β-catenin signaling pathway has been reported in many studies[18,19]. We performed the western blot to determine the expression levels of a gene related to Wnt/β-catenin signaling pathway. Our results showed that these protein expressions were also down-regulated in si-NEAT1 transfected NSCLC cells. In the current study, we found that the effect of NEAT1 on the Wnt/β-catenin pathway, which may be a potential therapeutic target to prevent NEAT1 metastasis and progression.

Conclusions

To our best knowledge, this was the first study to report that NEAT1 promoted the proliferation and metastasis of NSCLC cells and the activity of the Wnt/β-catenin signaling pathway. These results provided new evidence of NEAT1 as a promising tumor gene therapeutic target for NSCLC patients.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Siegel R, Ward E, Brawley O, Jemal A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA Cancer J Clin 2011; 61: 212-236.
2) Rosell R, Karachaliou N. Lung cancer: Maintenance therapy and precision medicine in NSCLC. Nat Rev Clin Oncol 2013; 10: 549-550.
3) Margaritora S, Cesario A, Cusumano G, Dall'armi V, Porziella V, Meacci E, Lococo F, D'Angelillo R, Congedo MT, Granone P. Pneumonectomy with and without induction chemo-radiotherapy for non-small cell lung cancer: short and long-term results from a single centre. Eur Rev Med Pharmacol Sci 2013; 17: 29-40.
4) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016; 66: 7-30.
5) Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155-159.
6) Tsai MC, Spitale RC, Chang HY. Long intergenic noncoding RNAs: new links in cancer progression. Cancer Res 2011; 71: 3-7.
7) Wang Z, Jin Y, Ren H, Ma X, Wang B, Wang Y. Downregulation of the long non-coding RNA TUSC7 promotes NSCLC cell proliferation and correlates with poor prognosis. Am J Transl Res 2016; 8: 680-687.
8) Li W, Sun M, Zang C, Ma P, He J, Zhang M, Huang Z, Ding Y, Shu Y. Upregulated long non-coding RNA AGAP2-AS1 represses LATS2 and KLF2 expression through interacting with EZH2 and LSD1 in non-small-cell lung cancer cells. Cell Death Dis 2016; 7: e2225.

9) Chen ZJ, Zhang Z, Xie BB, Zhang HY. Clinical significance of up-regulated lncRNA NEAT1 in prognosis of ovarian cancer. Eur Rev Med Pharmacol Sci. 2016; 20: 3373-3377.

10) He C, Jiang B, Ma J, Li Q. Aberrant NEAT1 expression is associated with clinical outcome in high grade glioma patients. APMIS 2016; 124: 169-174.

11) Wilusz JE. Long noncoding RNAs: Re-writing dogmas of RNA processing and stability. Biochim Biophys Acta 2016; 1859: 128-138.

12) Jiang P, Wu X, Wang X, Huang W, Feng Q. NEAT1 upregulates EGCG-induced CTR1 to enhance cisplatin sensitivity in lung cancer cells. Oncotarget 2016; 7: 43337-43351.

13) Ma Y, Liu L, Yan F, Wei W, Deng J, Sun J. Enhanced expression of long non-coding RNA NEAT1 is associated with the progression of gastric adenocarcinomas. World J Surg Oncol 2016; 14: 41.

14) Chen X, Kong J, Ma Z, Gao S, Feng X. Up regulation of the long non-coding RNA NEAT1 promotes esophageal squamous cell carcinoma cell progression and correlates with poor prognosis. Am J Cancer Res 2015; 5: 2808-2815.

15) Li Z, Wei D, Yang C, Sun H, Lu T, Kuang D. Overexpression of long noncoding RNA, NEAT1 promotes cell proliferation, invasion and migration in endometrial endometrioid adenocarcinoma. Biomed Pharmacother 2016; 84: 244-251.

16) Zhen L, Yun-Hui L, Hong-Yu D, Jun M, Yi-Long Y. Long noncoding RNA NEAT1 promotes glioma pathogenesis by regulating miR-449b-5p/c-Met axis. Tumour Biol 2016; 37: 673-683.

17) Sun C, Li S, Zhang F, Xi Y, Wang L, Bi Y, Li D. Long non-coding RNA NEAT1 promotes non-small cell lung cancer progression through regulation of miR-377-3p-E2F3 pathway. Oncotarget 2016; 7: 51784-51814

18) Xiao C, Wu CH, Hu HZ. LncRNA UCA1 promotes epithelial-mesenchymal transition (EMT) of breast cancer cells via enhancing Wnt/beta-catenin signaling pathway. Eur Rev Med Pharmacol Sci 2016; 20: 2819-2824.

19) Fu X, Li H, Liu C, Hu B, Li T, Wang Y. Long noncoding RNA AK126698 inhibits proliferation and migration of non-small cell lung cancer cells by targeting Frizzled-8 and suppressing Wnt/β-catenin signaling pathway. Onco Targets Ther 2016; 9: 3815-3827.