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Original Article

Long noncoding RNA TINCR promoted cell proliferation through sponging miR-29b in non-small cell lung cancer

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Abstract: Long noncoding RNA TINCR acts as a master regulator in somatic differentiation development and gastric cancer cells proliferation. However, it is still unclear whether lncRNA TINCR is also involved in the progression ofnon-small cell lung cancer. In this study, we tested TINCR expression in NSCLC tissues and cell linesby using real-time PCR. We silenced TINCR expressionby using siRNAtransfection, studiedcell proliferation by using EdUassay and nude mice model, examined the association of TINCR and miR-29b by using luciferase reporter assay.We observed that TINCR was highly expressed in NSCLC tissues and cells. While silencing TINCR expression inhibited cell proliferation in vitro. Additionally, expression of miR-29b was negatively correlated with TINCR in clinical NSCLC tissues. Moreover, miR-29b is a TINCR-targeting miRNA. Further studies showed that knockdown of TINCR inhibited NSCLC cell growth in vitro and inhibited tumorigenesis of NSCLC cells in vivo by targeting miR-29b, indicating that TINCR may be the potential therapeutic target for treating NSCLC.

Keywords: TINCR, miR-29b, proliferation, NSCLC

Introduction

Non-small cell lung cancer (NSCLC), including adenocarcinoma and squamous cell carcinoma, is the most common type of cancer and a leading cause of cancer-related death worldwide [1]. Most NSCLC patients are diagnosed at the advanced stages as they are usually asymptomatic at early stages [2]. Despite the fact that great advances have been made in surgical techniques and chemoradiation therapy, the prognosis of lung cancer remains to be dismal, with a 5-year survival rate of about 15% [3]. Therefore, it is important to reveal the molecular mechanisms of the development of NSCLC.

LncRNAs, a subgroup of non-protein coding transcripts, ranged in length from 200 bp to tens of kilobases (kb), and accumulating evidences have shown that lncRNAs may promote the formation and progression of NSCLC, such as LINC00473 [4], NEAT1 [5], AGAP2-AS1 [6], TATDN1 [7], and LINC01133 [8]. Sun et al. reported NEAT1 functioned as an oncogene in NSCLC by acting as a competing endogenous RNA (ceRNA) for miR-377-3p, and then leading tode-suppression of E2F3, a endogenous target of miR-377-3p, which was a core oncogene in promoting NSCLC progression [5]. But up to date, the detail molecular mechanisms of lncRNAs in NSCLC are still needed to be further clarified.

Terminal differentiation-induced ncRNA (TINCR), alncRNA required for induction of key differentiation genes in epidermal tissue, is downregulated in human squamous cell carcinoma [9-11]. In addition, TINCR is strongly up-regulated in human gastric carcinoma, where it was found to contribute to oncogenesis and cancer progression by regulating cell proliferation and apoptosis [12]. However, whether TINCR also acts as the hallmark of NSCLC is still unknown and needed to be studied.

In our study, we are committed to explore the underlying molecular mechanisms of TINCR on NSCLC progression. Interestingly, we found that TINCR is highly up-regulated in NSCLC tissues and cell lines. While silencing TINCR expression inhibited cell proliferation in vitro. Additionally, TINCR expression was conversely

with the expression of miR-375 in tumor- and adjacent normal tissue samples from 37 patients with NSCLC. Moreover, miR-29b is a TINCR-targeting miRNA by luciferase reporter assay. Further studies showed that knockdown of TINCR inhibited NSCLC cell growth in vitro and suppressed tumorigenesis of NSCLC cells in vivo by targeting miR-29b. Taken together, these data contribute to the understanding of the roles and molecular mechanisms of TINCR in NSCLC progression.

Materials and methods

Clinical ethics

Patient consent and Institutional Research Ethics Committee approval were obtained prior to the use of these clinical materials for research purposes.

Patients tissue samples

Paired NSCLC tissues and adjacent normal tissues were obtained from 37 NSCLC patients who underwent surgery for excision of a primary tumor in the department of Thoracic Surgery. All specimens were snap-frozen and stored at -80°C until total RNAextraction. Total RNAs were isolated by TRIzol(Invitrogen, Carlsbad, CA) extraction, according to the instructions of the manufacturer. All tumor and paired normal tissues were confirmed by experienced pathologists. Informed written consents were obtained from all patients included in this study.

Cell culture

The human non-small cell lung cancer cell lines A549, H1299, and H1650 and the human large cell lung cancer cells H460 were maintained in RPMI medium 1640 with 10% FBS and antibiotics (100 units/ml penicillin and 100 g/ml streptomycin). These cell lines were obtained from the Chinese Academy of Sciences (Shanghai, China).

Cell transfection

The human NSCLC cell lines A549 and H1299 were transfected with miR-29b mimic, inhibitor and scramble (Genepharma, Shanghai, China). miR-29b mimic, inhibitor and scramble at a final concentration 50 nM were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA) into cells seeded onto 6 well plates.

RNA interference

Small interfering RNAs (siRNAs) were chemically synthesized (Invitrogen, Shanghai, China). Transfection of siRNA duplexes into NSCLC cells was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 h post-transfection, cells were harvested for RT-PCR analysis.

Real-time PCR

Total RNA was extracted from human NSCLC tissues and cell lines by using of Trizol reagent (Invitrogen) according to its manufacture's instruction. RNA quality was measured by a spectrophotometer (Thermo Fisher). cDNA was synthesized by using of 1 ug of RNA and a commercially available kit (iScriptTM) according to the manufacture's instruction. Real-time PCR was performed using an instrument of ABI 7500 PCR (Applied Biosystems, Japan). The relative amount of mRNA was calculated using $2^{-\Delta Ct}$ method. Gene expression was normalized by β-actin. All data were obtained from three individual experiments.

Luciferase reporter assay

LncRNA TINCR fragment containing the predicted miR-29b binding site, the putative sequences of the binding site then cloned into a pmiR-GLO Dual-luciferase miRNA target expression vector to form the reporter vector pmiRGLO-TINCR-wild-type (TINCR-wt). To mutate the putative binding site of miR-29b in TINCR, the sequence of putative binding site was replaced as indicated and was named as pmiRGLO-TINCR-mutated-type (TINCR-mut). The pmiRGLO-TINCR-wt or pmiRGLO-TINCR-mut was cotransfected with miR-29b mimic or miR-29b NC into GC cells using Lipofectamie 2000 (Invitrogen, USA). After 48 h transfection the luciferase assay was used a Dual-Luciferase Reporter Assay System (Promega, WI, USA) according to the manufacturer's protocol.

Cell proliferation assay

To assess the effect of TINCR expression on cell proliferation in the human NSCLC cell lines A549 and H1299, the EdU (5'-ethynyl-2'-deoxyuridine) incorporation assay was performed to test cell proliferation using the Cell-Light™ EdU DNA Cell Proliferation Kit (Guangzhou Ribobio

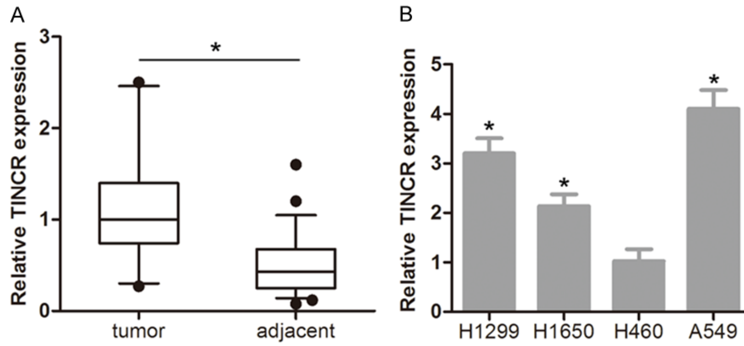

Figure 1. TINCR expression was increased in NSCLC. A. TINCR expression was examined by real-time PCR in 37 paired human NSCLC tissues and adjacent noncancerous tissues. B. Real-time PCR analysis of TINCR expression in the human large cell lung cancer cells (H460) and NSCLC cells. Statistically significant differences are indicated: **P* < 0.05; Student's t test.

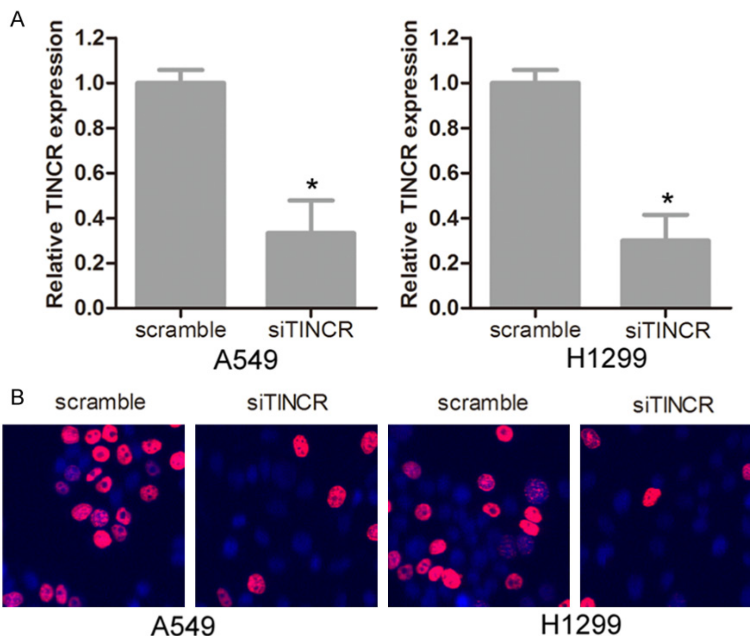

Figure 2. The knockdown of TINCR inhibited the proliferation of NSCLC cells. A. We transfected cells with siTINCR and scrambled siRNA for 24 hours, and detected the knockdown effect by real-time PCR. Statistically significant differences are indicated: **P* < 0.05; Student's t test. B. Growth curves of A549 and H1299 cells after transfection with siTINCR or scrambled siRNA were determined by EdU assays.

Co., Ltd, Guangzhou, China) according to the manufacturer's instructions.

Nude mice model

All animal work was conducted under the institutional guidelines and approved by the Use Committee for Animal Care. Nude mice were purchased from the Vital River Laboratories (Beijing, China). 5 × 10^6 A549 cells were transfected with miR-29b inhibitor, siTINCR or control in replicate. Before injection, cells were pooled and mixed with matrigel (BD Biosciences, USA). 1 × 10^6 cells were injected subcutaneously into the right flank of nude mice. Measurements were taken at day 6, 10, 14, 18, 22. And tumor volumes were calculated using the formula V = length × width ^ 2/2.

Statistical analysis

Data are presented as means ± SD. of three independent experiments. Differences between groups were analysed by GraphPad Prism 5 software (GraphPad Software, CA, USA) with Student's t-test or ANOVA. Differences were considered statistically significant at $P < 0.05$.

Results

TINCR was upregulated in NSCLC tissues and cell lines

TINCR expression was significantly higher ($P < 0.05$) in the NSCLC tumor tissues than in the adjacent normal tissues, as investigated using real-time PCR assays (**Figure 1A**). TINCR expression was also detected in the NSCLC cell lines, including A549, H1299 and H1650 and the human large cell lung cancer cells H460. Significantly high TINCR expression was found in A549, H1299 and H1650 compared with that in H460 ($P < 0.05$) (**Figure 1B**). The results suggested that the TINCR expression in NSCLC were significantly upregulated compared to adjacent normal tissues.

Knockdown of TINCR inhibited proliferation of NSCLC cell lines

To explore the function of TINCR in the regulation of cell proliferation, small interfering RNA

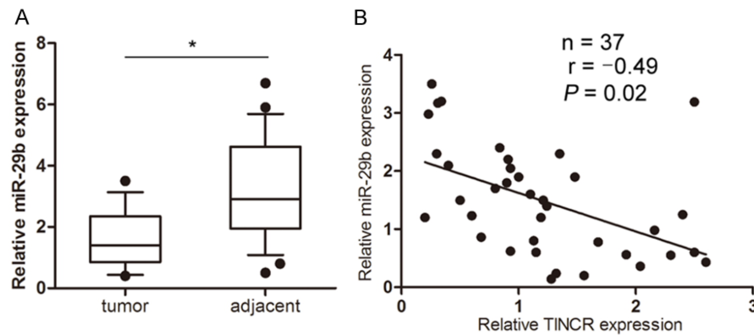

Figure 3. miR-29b was negatively associated with TINCR in clinical NSCLC tissues. A. miR-29b mRNA levels was examined by RT-PCR in 37 cases of clinical NSCLC tissues and adjacent normal tissues. B. Correlation of miR-29b levels with TINCR mRNA levels was examined by RT-PCR in 37 cases of clinical NSCLC tissues (Pearson's correlation coefficient, r = -0.49). Statistically significant differences are indicated: *P < 0.05; Student's t test.

(siRNA) was transfected into A549 and H1299 cells. TINCR expression was downregulated with TINCR siRNA transfection compared to the scrambled siRNA in A549 and H1299 cells, determined by RT-PCR (P < 0.05) (**Figure 2A**). The effect of TINCR expression on cell proliferation was evaluated by EdU assay. Knockdown of TINCR by TINCR siRNA transfection reduced the proliferation capacity of A549 and H1299 cells other than treated with scrambled siRNA (P < 0.05) (**Figure 2B**).

Expression of miR-29b was negatively correlated with TINCR in clinical NSCLC tissues

Then, we analyzed the expression of miR-29b in 37 paired clinical NSCLC and adjacent normal tissues using RT-PCR and normalized against an endogenous control (β-actin). MiR-29b expression was substantial decreased in 37NSCLC patient's tissues compared with corresponding adjacent tissues (P < 0.05) (**Figure 3A**). Then we assessed the correlation between TINCR and miR-29b. As expected, we found that the levels of miR-29b exhibited a significant negative correlation with the levels of TINCR mRNA (r = -0.49, P = 0.02) (**Figure 3B**). Thus, our finding indicated that the levels of miR-29b negatively associated with TINCR mRNA in NSCLC tissues.

TINCR acted as a molecular sponge for miR-29b in NSCLC cells

Recent studies have been reported that TINCR functioned as ceRNAs to specific miRNAs involved in tumor progression. We predicted miRNA target sites using online software program RNA hybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) and found out miR-29b with relevant binding sites in TINCR in both programs. According to the prediction results, there existed a putative miR-29b binding site in TINCR (**Figure 4A**). Additionally, the expression of TINCR downregulated by transfection with siTINCR led to substantial increased expression of miR-29b in both A549 and H1299 cells (P < 0.05) (**Figure 4B**). The results indicate that TINCR may play a role in deregulation of miR-29b. For further verification, the luciferase activity was detected after co-transfecting with pmiRGLO-TINCR-wt and miR-29b mimic or control, pmiRGLO-TINCR-mut and miR-29b mimic or negative control in NSCLC cells. The results clearly indicated that miR-29b mimic significantly suppressed the activity of pmiRGLO-TINCR-wt, but has no effect on the activity of pmiRGLO-TINCR-mut in both A549 and H1299 cells (P < 0.05) (**Figure 4C**), supporting that miR-29b is a TINCR-targeting miRNA.

TINCR acted as tumor oncotarget by targeting miR-29b in NSCLC

To determine the importance of miR-29b binding in TINCR promoting NSCLC progression, we downregulated miR-29b expression with transfection of miR-29b inhibitors in knockdown of TINCR A549 and H1299 cells, and the EdU assay revealed that low-expression of miR-29b weakened the inhibition effect of TINCR (P < 0.05) (**Figure 5A**). For further verification, we next evaluated tumor growth of xenografts derived from A549 cells that were co-transfecting with siTINCR and miR-29b inhibitor or scramble to subcutaneous injection into nude mice, and found that tumor growth suppression was abrogated with miR-29b down-regulated in TINCR knockdown of NSCLC cells xenografts (P < 0.05) (**Figure 5B**, **5C**). From the results we have showed, we concluded that Long noncoding RNA TINCR promoted cell proliferation through sponging miR-29b in non-small cell lung cancer.

Discussion

LncRNAs are implicated in pathologic and physiologic processes in numerous of human dis-

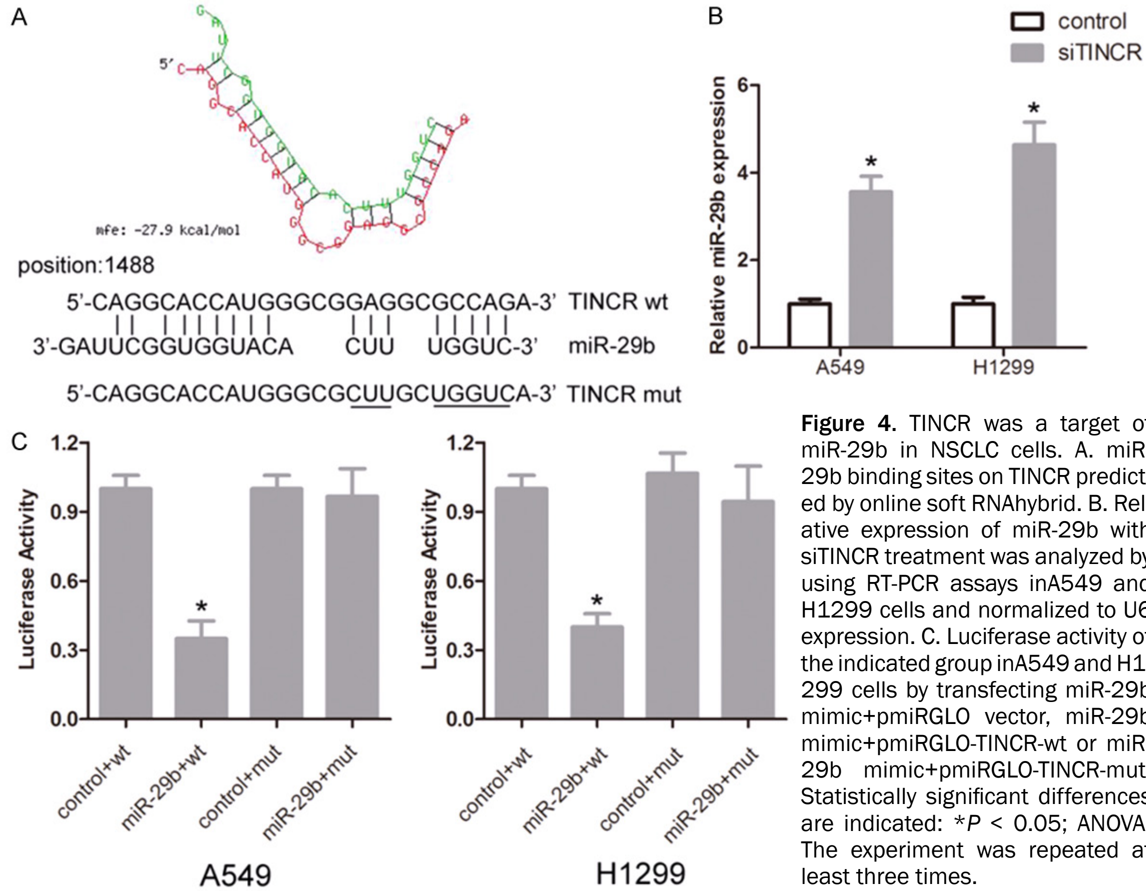

Figure 4. TINCR was a target of miR-29b in NSCLC cells. A. miR-29b binding sites on TINCR predicted by online soft RNAhybrid. B. Relative expression of miR-29b with siTINCR treatment was analyzed by using RT-PCR assays inA549 and H1299 cells and normalized to U6 expression. C. Luciferase activity of the indicated group inA549 and H1-299 cells by transfecting miR-29b mimic+pmiRGLO vector, miR-29b mimic+pmiRGLO-TINCR-wt or miR-29b mimic+pmiRGLO-TINCR-mut. Statistically significant differences are indicated: *P < 0.05; ANOVA. The experiment was repeated at least three times.

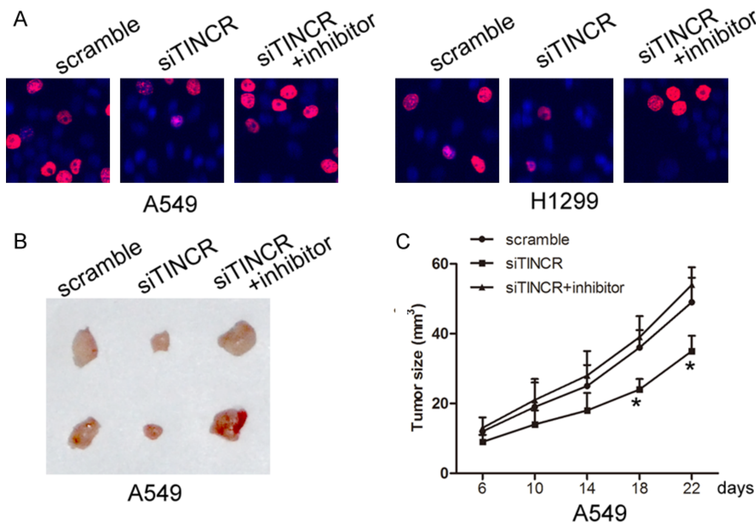

Figure 5. Knockdown of TINCR inhibited cell and tumor growth by targeting miR-29b. A. EdU cell proliferation assays were analyzed by transfecting scrambled siRNA, siTINCR and siTINCR+miR-29b inhibitor in A549 and H1299 cells. B. A549 cells were transfected with scrambled siRNA, siTINCR or siTINCR+miR-29b inhibitor and injected subcutaneously into 10 nude mice per flank. Surgical resections of A549 xenograft tumors on week 4 for animals were shown. C. Measurements of tumor volumes were taken weekly and tumor volumes were shown. Statistically significant differences are indicated: *P < 0.05; ANOVA. The experiment was repeated at least three times.

eases. The levels of certain lncRNAs are associated with the metastasis, development, and prognosis of cancers [5, 13-15]. Given that abnormal expression of TINCR showed tumor oncotarget or suppressor via involving in cell proliferation, migration and invasion, and apoptosis in cancers [12, 16, 17], We speculated that TINCR might involve in the NSCLC progression. In the present study, we identified TINCR as an oncogenic lncRNA in NSCLC by analyzing in 37 pairs NSCLC cancer and adjacent normal tissues revealed that TINCR is up-regulated in NSCLC tissues. Moreover, silencing TINCR inhibited NSCLC cell growth in vitro and inhibited tumorigenesis of NSCLC cells in vivo.

Generally, lncRNA involved in regulation of cancer cells phenotypes by regulating target gene expression by different mechanisms, including chromatin modification, genomic imprinting, RNA decay and sponging miRNAs. A study reported that loss of TINCR expression promoted proliferation, metastasis through activating EpCAM cleavage in colorectal cancer [17]. TINC Rregulated cell proliferation and apoptosis by affecting KLF2 mRNA stability in gastric cancer 25728677. While whether TINCR could act as a ceRNA by sponging miRNA in NSCLC cells is unknown. Interestingly, we found that TINCR acted as a molecular sponge for miR-29b in NSCLC cells, which suggesting that TINCR might be involved in the progression of NSCLC by targeting miR-29b. In previous study, miR-29b was down-regulated and attenuated non-small cell lung cancer metastasis and promote tumor aggressiveness [18, 19]. Ourfurther study showed that miR-29b could function as tumor suppressor and its' expression could be suppressed by TINCR in cells.

In conclusion, our data clarified that the expression of TINCR is substantial increased in NSCLC tissues and cell lines. Moreover, TINCR knockdown repressed cell proliferation in NSCLC cell lines. Furthermore, TINCR acted as a ceRNA by sponging miR-29b inhibited NSCLC cell growth in vitro and tumorigenesis of NSCLC cells in vivo. Our findings firstly elucidated the tumor-oncogenic role of TINCR in NSCLC, and indicated that TINCR could be a potential therapeutic target in NSCLC.

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Disclosure of conflict of interest

None.

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References

[1] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 1: 11-30.

[2] Boolell V, Alamgeer M, Watkins DN, Ganju V. The evolution of therapies in non-small cell lung cancer. Cancers (Basel) 2015; 3: 1815-1846.

[3] Vijayvergia N, Shah PC, Denlinger CS. Survivorship in non-small cell lung cancer: challenges faced and steps forward. J Natl Compr Canc Netw 2015; 9: 1151-1161.

[4] Chen Z, Li JL, Lin S, Cao C, Gimbrone NT, Yang R, Fu DA, Carper MB, Haura EB, Schabath MB, Lu J, Amelio AL, Cress WD, Kaye FJ, Wu L. cAMP/CREB-regulated LINC00473 marks LKB1-inactivated lung cancer and mediates tumor growth. J Clin Invest 2016; 6: 2267-2279.

[5] 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; 32: 51784-51814.

[6] 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.

[7] Zequn N, Xuemei Z, Wei L, Zongjuan M, Yujie Z, Yanli H, Yuping Z, Xia M, Wei W, Wenjing D, Na F, Shuanying Y. The role and potential mechanisms of LncRNA-TATDN1 on metastasis and invasion of non-small cell lung cancer. Oncotarget 2016; 14: 18219-18228.

[8] Zang C, Nie FQ, Wang Q, Sun M, Li W, He J, Zhang M, Lu KH. Long non-coding RNA LINC01133 represses KLF2, P21 and E-cadherin transcription through binding with EZH2, LSD1 in non small cell lung cancer. Oncotarget 2016; 10: 11696-11707.

[9] Sun BK, Boxer LD, Ransohoff JD, Siprashvili Z, Qu K, Lopez-Pajares V, Hollmig ST, Khavari PA. CALML5 is a ZNF750- and TINCR-induced protein that binds stratifin to regulate epidermal differentiation. Genes Dev 2015; 21: 2225-2230.

[10] Kretz M, Siprashvili Z, Chu C, Webster DE, Zehnder A, Qu K, Lee CS, Flockhart RJ, Groff AF, Chow J, Johnston D, Kim GE, Spitale RC, Flynn RA, Zheng GX, Aiyer S, Raj A, Rinn JL, Chang HY, Khavari PA. Control of somatic tissue differentiation by the long non-coding RNA TINCR. Nature 2013; 7431: 231-235.

[11] Lopez-Pajares V, Qu K, Zhang J, Webster DE, Barajas BC, Siprashvili Z, Zarnegar BJ, Boxer LD, Rios EJ, Tao S, Kretz M, Khavari PA. A LncRNA-MAF: MAFB transcription factor network regulates epidermal differentiation. Dev Cell 2015; 6: 693-706.

[12] Xu TP, Liu XX, Xia R, Yin L, Kong R, Chen WM, Huang MD, Shu YQ. SP1-induced upregulation of the long noncoding RNA TINCR regulates cell proliferation and apoptosis by affecting KLF2 mRNA stability in gastric cancer. Oncogene 2015; 45: 5648-5661.

[13] Chen N, Guo D, Xu Q, Yang M, Wang D, Peng M, Ding Y, Wang S, Zhou J. Long non-coding RNA FEZF1-AS1 facilitates cell proliferation and migration in colorectal carcinoma. Oncotarget 2016; 10: 11271-11283.

[14] Lu Z, Xiao Z, Liu F, Cui M, Li W, Yang Z, Li J, Ye L, Zhang X. Long non-coding RNA HULC promotes tumor angiogenesis in liver cancer by up-regulating sphingosine kinase 1 (SPHK1). Oncotarget 2016; 1: 241-254.

[15] Ma Y, Yang Y, Wang F, Moyer MP, Wei Q, Zhang P, Yang Z, Liu W, Zhang H, Chen N, Wang H, Qin H. Long non-coding RNA CCAL regulates colorectal cancer progression by activating Wnt/beta-catenin signalling pathway via suppression of activator protein 2alpha. Gut 2016; 9: 1494-1504.

[16] Chen Z, Liu Y, He A, Li J, Chen M, Zhan Y, Lin J, Zhuang C, Liu L, Zhao G, Huang W, Cai Z. Theophylline controllable RNAi-based genetic switches regulate expression of lncRNA TINCR and malignant phenotypes in bladder cancer cells. Sci Rep 2016; 6: 30798.

[17] Zhang ZY, Lu YX, Chang YY, Zheng L, Yuan L, Zhang F, Hu YH, Zhang WJ, Li XN. Loss of TINCR expression promotes proliferation, metastasis through activating EpCAM cleavage in colorectal cancer. Oncotarget 2016; 16: 22639-22649.

[18] Wu DW, Hsu NY, Wang YC, Lee MC, Cheng YW, Chen CY, Lee H. c-Myc suppresses microRNA-29b to promote tumor aggressiveness and poor outcomes in non-small cell lung cancer by targeting FHIT. Oncogene 2015; 16: 2072-2082.

[19] Wang H, Guan X, Tu Y, Zheng S, Long J, Li S, Qi C, Xie X, Zhang H, Zhang Y. MicroRNA-29b attenuates non-small cell lung cancer metastasis by targeting matrix metalloproteinase 2 and PTEN. J Exp Clin Cancer Res 2015; 34: 59.