Long non-coding RNA-NEF targets glucose transportation to inhibit the proliferation of non-small-cell lung cancer cells

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Received March 14, 2018; Accepted October 29, 2018

DOI: 10.3892/ol.2019.9919

Abstract. Long non-coding RNA (IncRNA)-NEF is a newly discovered IncRNA, which exhibits an inhibitory function on the metastasis of hepatocellular carcinoma, while its involvement in other types of malignancy are unknown. In the present study, tumor and adjacent healthy tissues were obtained from patients with non-small-cell lung cancer (NSCLC), and blood was obtained from patients with NSCLC and healthy individuals. Expression levels of IncRNA-NEF in tumor tissue samples, healthy tissue samples and serum were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Receiver operating characteristic curve analysis and survival curve analysis were performed to evaluate the diagnostic and prognostic value of serum IncRNA-NEF for NSCLC. The effects of IncRNA-NEF overexpression in NSCLC cell lines on tumor cell proliferation, glucose uptake, glucose transporter 1 (GLUT1) protein expression and mRNA expression were investigated by Cell Counting kit-8 assay, glucose uptake assay, western blot analysis and RT-qPCR, respectively. It was identified that IncRNA-NEF was downregulated in NSCLC tissues, compared with healthy controls, and the serum level of IncRNA-NEF was negatively associated with primary tumor stage. Therefore, serum IncRNA-NEF may be a sensitive diagnostic and prognostic marker for NSCLC. Overexpression of IncRNA-NEF inhibited NSCLC cell proliferation and glucose uptake, and downregulated GLUT1 expression. Therefore, it can be concluded that IncRNA-NEF can target glucose transportation to inhibit the proliferation of NSCLC cells.

Introduction

Lung cancer is one of the most common types of human malignancy and is also one of the leading causes of cancer-associated mortality (1). In developing countries, including China, environmental pollution has resulted in increased incidence and mortality rates of lung cancer in the past 10 years (2). Additionally, the incidence rate of lung cancer is predicted to increase in the future (2). Non-small-cell lung cancer (NSCLC) is a major type of lung cancer and accounts for ~85% of all cases worldwide (3). Despite efforts to develop treatment strategies, surgical resection remains the only radical treatment for NSCLC (4). However, due to a lack of typical symptoms at the early stage of NSCLC, the majority of patients with NSCLC are diagnosed at an advanced stage, when surgical resection is inappropriate (5). Therefore, early diagnosis and treatment for patients with NSCLC is critical to improve the survival rate.

The human genome not only transcribes mRNAs, which encode protein products, but also transcribes a large set of non-coding RNAs (ncRNAs), which serve key roles in almost all critical physiological and pathological processes (6). Long ncRNAs (IncRNAs) are a subgroup of ncRNAs that are composed of >200 nucleotides (7). It has been demonstrated that various IncRNAs, such as IncRNA PVT1 and IncRNA MEG, serve a number of roles in the onset, development and progression of NSCLC (8,9). IncRNA-NEF is a novel IncRNA, which exhibits a critical function in hepatocellular carcinoma (10). Cancer development is characterized by the accelerated glucose metabolism, which provided energy for cancer development and progression (11). Glucose transporter 1 (GLUT1) is as a key player in glucose uptake also participates in cancer biology (12). The present study investigated the role of IncRNA-NEF in NSCLC and revealed that IncRNA-NEF can target glucose transportation, more specifically GLUT1, to inhibit the proliferation of NSCLC cells. The observation of the present study provides novel insights into the diagnosis and treatment strategies of NSCLC.

Patients and methods

Patients. The present study included 86 patients with NSCLC. All patients were pathologically diagnosed with NSCLC and treated at the First Hospital of Jilin University (Jilin, China) from July 2010 to January 2012. The total 86 patients included 52 males and 34 females, with an age range of 20-74 years and a mean age of 45.2±10.2 (standard deviation) years. Patients with another critical disease, another lung disease or a mental disorder were excluded from the study. Primary tumors were staged according to the following criteria: Tis,
tumor in situ, 12 cases; T1, tumor ≤3 cm in greatest dimension, 14 cases; T2, tumor >3 cm and ≤5 cm in greatest dimension, 19 cases; T3, tumor >5 and ≤7 cm in greatest dimension, 20 cases; and T4, tumor >7 cm in greatest dimension, 21 cases. Additionally, 44 healthy individuals with similar age and sex distributions were included to serve as a control group. The control group included 30 males and 14 females, with an age range of 22-70 years and a mean age of 46.1±8.9 years. The present study was approved by The Ethics Committee of the First Hospital of Jilin University. All patients signed informed consent.

Sample collection. Tumor and adjacent healthy tissue samples (within 5 cm of the tumor) were collected from 33 patients during surgery. All tissue samples were 100-200 mg. Blood (10 ml) was also extracted from the elbow vein of the 86 patients and 44 healthy controls. Serum was separated from the blood of the remaining 55 patients by incubating the blood at room temperature for 2 h, followed by centrifugation at 1,000 x g at room temperature for 20 min. All samples were stored in liquid nitrogen (-196°C) prior to use.

Cell lines and cell culture. The following human NSCLC cell lines were purchased from American Type Culture Collection (Manassas, VA, USA): NCI-H23 (lung adenocarcinoma), NCI-H522 (lung adenocarcinoma), NCI-H520 (squamous cell carcinoma) and NCI-H2170 (squamous cell carcinoma). All cell lines were cultured in RPMI-1640 medium (cat. no. ATCC 30-2001) containing 10% fetal bovine serum (cat. no. ATCC 30-2020; both American Type Culture Collection) at 37°C in a 5% CO₂ incubator. Cells were harvested during the logarithmic growth phase for subsequent experiments.

Construction of IncRNA-NEF-overexpressing cell lines. NEF complementary DNA (cDNA) surrounded by ECOR I cutting sites was obtained through polymerase chain reaction (PCR) amplification, which was performed by Sangon Biotech Co., Ltd., (Shanghai, China). A IncRNA-NEF overexpression vector was established by inserting NEF cDNA into a ECOR I linearized pIRE2-EGFP vector (Clontech Laboratories Inc., Mountainview, CA, USA). NCI-H23, NCI-H522, NCI-H520 and NCI-H2170 cell lines were cultured overnight at 37°C to reach 80-90% confluence and Lipofectamine® 2000 (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect 10 nM IncRNA-NEF overexpression vector or 10 nM empty pIRE2-EGFP vector (negative control) into 5x10⁴ cells. Lipofectamine 2000 and the DNA was mixed and kept at room temperature for 20 min to allow the formation of reagent-DNA complexes. The complexes were then incubated with the cells at 37°C for 5-6 h to achieve transfection. Subsequently, the transfection mixture was immediately replaced with RPMI-1640 medium (37°C) to avoid toxic effects.

Cell proliferation assay. Transfected cells of all cell lines were collected during the logarithmic growth phase and a suspension with a cell density of 4x10⁴ cells/ml was generated using RPMI-1640 medium. Subsequently, 100 μl cell suspension was added to each well of a 96-well plate. Cell Counting kit-8 (CCK-8, Sigma-Aldrich, Merck KGaA) solution (10 μl) was added to each well 24, 48, 72 and 96 h later. Following incubation at 37°C for a further 4 h, the optical density value of each well at 450 nm was measured using a Fisherbrand accuSkan GO UV/Vis Microplate Spectrophotometer (Thermo Fisher Scientific, Inc.). OD values of control group at 9 h were set to 100, and other groups or other time points were normalized to the control group at 96 h.

Glucose uptake assay. Transfected cells of all cell lines were collected during the logarithmic growth phase and a cell suspension with a cell density of 4x10⁴ cells/ml was generated. Subsequently, 10 ml cell suspension (4x10⁷ cells) was prepared using RPMI-1640 medium was added into each well of a 6-well plate. Following incubation for 24 h at 37°C, the cells were washed with PBS once and incubated with 2 ml Krebs-Ringer-HEPES (KRH) buffer (120 mM NaCl, 25 mM Heps, pH 7.4, 1.2 mM MgSO₄, 5 mM KCl, 1.3 mM CaCl₂ and 1.3 mM KH₂PO₄) containing 1 μCi [3H]-2-deoxyglucose (PerkinElmer, Inc., Waltham, MA, USA) at 37°C for 20 min. Subsequently, pre-cooled KRH buffer was used to wash the cells once and block glucose uptake. Finally, cells were mixed with 300 μl lysis buffer (0.2% SDS and 10 mM Tris-HCl, pH 8.0) and radioactivity was measured by liquid scintillation spectrometry. Disintegrations per minute was used to represent the intracellular level of [3H]-2-deoxyglucose.

Reverse transcription-quantitative PCR. Tumor and adjacent healthy tissue samples were ground in liquid nitrogen, followed by addition of TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) to extract total RNA. TRIzol® reagent was also directly mixed with serum and in vitro cultivated cells to extract total RNA. RNA quality was assessed using a NanoDrop™ 2000 Spectrophotometer (Invitrogen; Thermo Fisher Scientific). RNA samples with an A260/A280 ratio between 1.8-2.0 were used to synthesize cDNA by reverse transcription using a SuperScript III Reverse Transcriptase kit (Thermo Fisher Scientific, Inc.). Reaction conditions were as follows: 25°C for 5 min, 50°C for 30 min and 85°C for 15 min. A PCR reaction system was prepared using SYBR®-Green Real-Time PCR Master mix (Thermo Fisher Scientific, Inc.) and the following primers were used: IncRNA-NEF forward, 5'-AGC CTC TAT 13'- and reverse, 5'-GCC CAA ACAGCTCTCCAAATT-3'; GLUT1 forward, 5'-AGG TGA CCA 13'- and reverse, 5'-TCA AAG GAC TTG CCC AGT TT-3'; 18S forward, 5'-GAA GAA CAC CCT GGA TCC-3' and reverse, 5'-GAC CCTTAT GCCAACACAGT-3' and human β-actin forward, 5'-GAC CTTCAT GCCAACACAGT-3' and reverse, 5'-AGTACCTGGCTCATG GAGGA-3'. PCR reaction conditions were as follows: 95°C for 45 sec, followed by 40 cycles of 10 sec at 95°C and 40 sec at 60°C. All data were quantified using the 2-ΔΔCT method (13). The relative expression level of IncRNA-NEF was normalized to the expression level of β-actin.

Western blot analysis. Total protein extraction from all in vitro cultured NSCLC cell lines was performed using radioimmuno-precipitation assay solution (Thermo Fisher Scientific, Inc.) and bicinechonic acid assay was used for protein quantification. Subsequently, 10% SDS-PAGE gel electrophoresis was performed with 30 μg protein per lane, followed by transfer to polyvinylidene difluoride membranes. Membranes were blocked with 5% skimmed milk for 2 h at room temperature,
followed by washing twice with TBS with 0.3% Tween (TBST) for 15 min each time. The membranes were then incubated with rabbit anti-GLUT1 primary (1:2,000; cat. no. ab15309; Abcam, Cambridge, UK) and rabbit anti-GAPDH primary antibodies (1:1,000; cat. no. ab8245; Abcam) overnight at 4°C. Subsequently, the membranes were washed twice with TBST for 15 min each time and further incubated with anti-rabbit IgG-horseradish peroxidase-labeled secondary antibody (1:1,000; cat. no. MBS435036; MyBioSource, Inc., San Diego, CA, USA) at room temperature for 1 h. Following washing twice with TBST for 15 min each time, enhanced chemiluminescent (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to develop a signal. Signals were detected using a MYECL™ Imager (Thermo Fisher Scientific, Inc.) and relative expression level of GLUT1 was normalized to endogenous control GAPDH using ImageJ v1.48 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. SPSS 19.0 (IBM Corp., Armonk, NY, USA) was used to perform all statistical analysis. All data are expressed as mean ± standard deviation. Comparisons between two groups and among multiple groups were performed by paired Student's t-test and one-way analysis of variance followed by Tukey post-hoc test, respectively. The Kaplan-Meier method was used to plot survival curves and survival curves were compared using a log rank t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of lncRNA-NEF is downregulated in tumor tissues, compared with adjacent healthy tissues in patients with NSCLC. The expression level of lncRNA-NEF in tumor tissues and adjacent healthy tissues obtained from 33 patients with NSCLC was detected by RT-qPCR. A significantly increased expression level of lncRNA-NEF was observed in adjacent tissues, compared with tumor tissues, for 30/33 patients with NSCLC (P<0.01; Fig. 1). By contrast, a significantly increased expression level of lncRNA-NEF was identified in tumor tissues, compared with adjacent tissues, for 1 patient with NSCLC (P<0.01; Fig. 1). No significant differences were revealed in the expression level of lncRNA-NEF in tumor tissues, compared with adjacent tissues, for 2 patients. These data demonstrate that downregulation of lncRNA-NEF is associated with the pathogenesis of NSCLC.

Levels of circulating lncRNA-NEF in serum are associated with the primary tumor stage. Levels of circulating lncRNA-NEF in the serum of patients with NSCLC and healthy controls were measured by RT-qPCR. Levels of serum lncRNA-NEF were significantly increased in healthy controls, compared with patients with all stages of NSCLC (P<0.05; Fig. 2). Additionally, levels of serum lncRNA-NEF were significantly negatively associated with an increase in primary tumor stage (Fig. 2). These data indicate that lncRNA-NEF is associated with the progression of NSCLC.

Diagnostic and prognostic value of circulating serum lncRNA-NEF for NSCLC. Receiver operating characteristic curve analysis was performed to evaluate the diagnostic value of serum lncRNA-NEF for NSCLC. The area under the curve was 0.9421 with a 95% confidence interval of 0.9073-0.9769 (P<0.0001; Fig. 3A). Patients were divided into high expression and low expression groups according to the median level of serum lncRNA-NEF. Follow-up was completed for 5 years for all patients to record the survival rates. The Kaplan-Meier method was used to plot survival curves for both expression groups and survival curves were compared using a log rank t-test. As depicted in Fig. 3B, the overall survival rate of patients with a high level of serum lncRNA-NEF was significantly increased, compared with the survival rate of patients.
In NSCLC, a major type of lung cancer, it has previously been reported that the development of numerous human malignancies is associated with altered expression patterns of certain lncRNAs, such as lncRNA PVT1 and lncRNA MEG (7). In a previous study, 47 lncRNAs were revealed to be differentially-expressed in normal lung and NSCLC tumor tissues, compared with healthy tissues (14). lncRNA-NEF is a novel lncRNA, which is downregulated in hepatocellular carcinoma (10). In the present study, lncRNA-NEF was identified to be significantly downregulated in NSCLC tumor tissues, compared with adjacent healthy lung tissues, in the majority of patients with NSCLC. Additionally, serum levels of lncRNA-NEF were significantly negatively associated with an increase in primary tumor stage, which indicates a possible involvement of lncRNA-NEF in tumor growth. Notably, it has previously been demonstrated that lncRNA-NEF exhibits no significant effect on hepatocellular carcinoma growth (10), indicating differences in the pathogenesis of NSCLC and hepatocellular carcinoma.

It is understood that early diagnosis and treatment is critical for the survival of patients with the majority of cancer types, including NSCLC. Development of human disease is typically accompanied with changing levels of certain substances in the blood, and detecting these changes may provide biomarkers for the diagnosis and treatment of certain diseases (15). The present study demonstrated that the level of serum lncRNA-NEF could be used to effectively distinguish patients with NSCLC from healthy controls. Additionally, a high level of serum lncRNA-NEF was associated with poor survival of patients. Therefore, lncRNA-NEF may serve as a diagnostic and prognostic biomarker of NSCLC, as well as a treatment target. lncRNA-NEF is a novel lncRNA with, to the best of our knowledge, an unknown expression pattern in other human diseases, except hepatocellular carcinoma (10). Therefore, multiple biomarkers may be combined to improve the accuracy of diagnosis and prognosis.

The present study revealed that lncRNA-NEF overexpression significantly promoted the proliferation of NSCLC cells. Glucose uptake and metabolism provide energy for the proliferation of both normal cells and cancer cells (16), and abnormally accelerated energy metabolism is considered a unique feature of cancer cells, compared with normal healthy cells (17). Therefore, energy metabolism can be

Discussion

The present study investigated the role of lncRNA-NEF in NSCLC, a major type of lung cancer. It has previously been revealed that lncRNA-NEF overexpression significantly promoted the proliferation of NSCLC cells. Glucose uptake and metabolism provide energy for the proliferation of both normal cells and cancer cells (16), and abnormally accelerated energy metabolism is considered a unique feature of cancer cells, compared with normal healthy cells (17). Therefore, energy metabolism can be

with a low level of serum lncRNA-NEF (P<0.001). These data indicate that the level of serum lncRNA-NEF may serve as a diagnostic and prognostic biomarker for NSCLC.

lncRNA-NEF overexpression inhibits proliferation of NSCLC cells. The aforementioned circulating lncRNA-NEF in serum results demonstrated that the expression level of lncRNA-NEF was negatively associated with tumor size (primary tumor stage), indicating an involvement of lncRNA-NEF in tumor growth. To further investigate the role of lncRNA-NEF in NSCLC growth, cell lines overexpressing lncRNA-NEF were established and confirmed by RT-qPCR (P<0.05; Fig. 4A). The effects of lncRNA-NEF on cell proliferation were investigated with a CCK-8 assay. As demonstrated in Fig. 4B, lncRNA-NEF overexpression significantly inhibited the proliferation of all four transfected cell lines, compared with control cells (P<0.05; Fig. 4B). This supports an inhibitory effect of lncRNA-NEF on NSCLC cell proliferation.

lncRNA-NEF overexpression inhibits glucose uptake by NSCLC cells. Glucose uptake and metabolism provides energy for growth of healthy cells and cancer cells. Therefore, the effects of lncRNA-NEF overexpression on glucose uptake in NSCLC cells were investigated. As demonstrated in Fig. 5A, the glucose uptake level was significantly upregulated in the four transfected cell lines, compared with control cells (P<0.05). GLUT1 serves a key role in glucose uptake and metabolism. As depicted in Fig. 5B and C, lncRNA-NEF overexpression significantly increased the GLUT1 protein and mRNA expression levels in all four transfected cell lines, compared with control cells (P<0.05). These data indicate that lncRNA-NEF may inhibit glucose uptake in NSCLC cells by downregulating the expression of GLUT1, which could inhibit the growth of NSCLC.

Figure 3. Diagnostic and prognostic value of serum lncRNA-NEF levels for NSCLC. (A) The diagnostic value of serum lncRNA-NEF level was analyzed by receiver operating characteristic curve analysis. (B) A comparison of survival curves for patients with high and low levels of serum lncRNA-NEF. Patients were divided into high expression and low expression groups according to the median level of serum lncRNA-NEF. Serum lncRNA-NEF level may serve as a potential diagnostic and prognostic marker for NSCLC. NSCLC, non-small-cell lung cancer; lncRNA, long non-coding RNA; AUC, area under the curve; CI, confidence interval.
regarded as a target for the treatment of different types of malignancy (18). As a major component of glucose uptake and metabolism, GLUT1 typically demonstrates upregulated expression during the development of numerous tumor types (19,20), including NSCLC. Upregulated expression of GLUT1 promotes tumor cell proliferation (21) and inhibits tumor cell death (22). In the present study, IncRNA-NEF overexpression significantly inhibited glucose uptake in four NSCLC cell lines and downregulated the expression of GLUT1 in these cells. These data indicate that IncRNA-NEF overexpression may inhibit glucose uptake and metabolism of NSCLC cells by downregulating the expression of GLUT1, which may exert an inhibitory effect on the tumorigenesis of NSCLC.

Figure 4. IncRNA‑NEF overexpression inhibits proliferation of NSCLC cells. (A) IncRNA‑NEF expression level following transfection was significantly increased, compared with control cells. (B) IncRNA‑NEF overexpression inhibited proliferation of all four NSCLC cell lines, including two lung adenocarcinoma cell lines, NCI-H23 and NCI-H522, and two squamous cell carcinoma cell lines, NCI-H520 and NCI-H2170. *P<0.05. Control, control cells without transfection; empty vector, negative control cells transfected with empty vector; NEF, cells transfected with IncRNA-NEF overexpression vector; NSCLC, non-small-cell lung cancer; IncRNA, long non-coding RNA. Experiments were performed in triplicate manner and data were expressed as mean ± standard deviation.
In conclusion, lncRNA-NEF was downregulated in NSCLC and a decrease in serum level of lncRNA-NEF was associated with an increasing size of primary tumor. Serum lncRNA-NEF is a sensitive diagnostic and prognostic marker for NSCLC. lncRNA-NEF overexpression inhibited NSCLC cell proliferation and glucose uptake, and downregulated GLUT1 expression. Therefore, it can be concluded that lncRNA-NEF targets glucose transportation to inhibit the proliferation of NSCLC cells. However, the present study is limited by the small sample size, and future studies with larger sample sizes are required to further confirm our conclusions.

Acknowledgements
Not applicable.