IncRNA-NEF is downregulated in osteosarcoma and inhibits cancer cell migration and invasion by downregulating miRNA-21

QINGLEI YANG1,2, HONGYING YU3, QINGSHUI YIN1,4, XIAOMING HU1 and CHENGCHENG ZHANG1

1Department of Orthopedics, Guangzhou School of Clinical Medicine, Southern Medical University, Guangzhou, Guangdong 510515; Departments of 2Orthopedics and 3Pharmacy, Yuebei People's Hospital, Shaoguan, Guangdong 512000; 4Department of Orthopedics, Guangzhou General Hospital of Guangzhou Military Command (Liuhuaqiao Hospital), Guangzhou, Guangdong 510515, P.R. China

Received September 8, 2018; Accepted March 15, 2019

DOI: 10.3892/ol.2019.10276

Abstract. Long non-coding RNA (IncRNA)-NEF inhibits cancer metastasis in hepatocellular carcinoma; however, its role in other malignancies is unknown. The present study revealed that plasma IncRNA-NEF was downregulated, while miRNA-21 was upregulated in patients with osteosarcoma compared with healthy controls. Plasma IncRNA-NEF and miRNA-21 were negatively correlated in patients with osteosarcoma and healthy controls. Downregulation of IncRNA-NEF and upregulation of miRNA-21 distinguished patients with osteosarcoma from healthy controls. IncRNA-NEF overexpression mediated the inhibition of miRNA-21 expression in osteosarcoma cell lines, while miRNA-21 overexpression did not significantly affect the expression of IncRNA-NEF. IncRNA-NEF overexpression inhibited, while miRNA-21 overexpression promoted, migration and invasion of osteosarcoma cell lines in vitro. miRNA-21 overexpression partially compensated the inhibitory effects of IncRNA-NEF on osteosarcoma cell migration and invasion. Therefore, IncRNA-NEF may inhibit cancer cell migration and invasion in osteosarcoma by downregulating miRNA-21.

Introduction

Osteosarcoma is a rare type of malignancy which originates in bone and affects ~5 in 1,000,000 patients (1). In spite of the low incidence, osteosarcoma has a high mortality rate due to the high prevalence rate of metastases by the time of first diagnosis (2). Improved treatment strategies have increased the survival time of patients with osteosarcoma (3,4). However, the overall five-year survival rate of patients with this disease remains ~30% (5). The mechanisms underlying the pathogenesis of osteosarcoma have not been fully elucidated, potentially hindering the development of novel treatment strategies for this disease (6). Therefore, in-depth studies on the molecular mechanisms involved in osteosarcoma may improve its treatment.

In addition to protein-coding genes, the human genome contains a large number of genes which are transcribed to non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) (7,8). miRNAs and lncRNAs participate in the regulation of cell growth, proliferation, differentiation, migration and invasion by interacting with a number of signaling pathways (7,8). Previous studies have revealed that non-coding RNAs serve key roles in a number of human diseases, including metabolic diseases, heart diseases and cancer (9,10). As an oncogenic miRNA, miRNA-21 promotes tumor development in different types of cancer, including osteosarcoma (11). miRNA-21 may participate in tumorigenesis through interactions with IncRNA (12). IncRNA neighboring enhancer of FOXA2 (IncRNA-NEF) is a recently identified tumor suppressor IncRNA which is involved in hepatocellular carcinoma (13). To the best of our knowledge, no studies investigating the interaction between IncRNA-NEF and miRNAs have been reported. The present study revealed that IncRNA-NEF may inhibit cancer cell migration and invasion in osteosarcoma by downregulating miRNA-21.

Materials and methods

Plasma specimens. Blood specimens (5 ml) were collected from 49 patients with osteosarcoma and 42 healthy volunteers who were admitted to Guangzhou General Hospital of Guangzhou Military Command (Guangzhou, China) between September 2015 and June 2018. Blood was placed in anticoagulation tubes and centrifuged at room temperature for 15 min at 2,000 x g to collect the plasma. The inclusion criteria of patients with osteosarcoma were as follows: i) Osteosarcoma was diagnosed by pathological examinations; ii) osteosarcoma stage I or II; iii) patients who were diagnosed and treated for the first time; and iv) patients who were willing to participate in the study. The exclusion criteria were as follows: i) Presence
of co-morbidities; and ii) patients who were treated for other clinical disorders in the three months prior to admission. The patient group included 26 males and 23 females, with an age range of 12-33 years and a mean age of 19.3±4.2 years. The control group was composed of 23 males and 19 females, with an age range of 13-30 years and a mean age of 18.8±4.7 years. The two groups had similar gender and age distributions. Ethical approval was obtained from the Ethics Committee of Guangzhou General Hospital of Guangzhou Military Command. The study followed the tenets of the Declaration of Helsinki, and written informed consent was obtained from all patients and controls after the nature and possible consequences of the study were explained.

Cell culture. The human osteosarcoma cell lines MG-63 and U2OS were used in the current study. The two cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Eagle's Minimum Essential Medium (cat no. 30-2003; ATCC) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sangon Biotech Co., Ltd., Shanghai, China) was used to cultivate cells in an incubator (37°C, 5% CO₂).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from plasma and cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and miRNAs were extracted from plasma and cells using the miRNeasy Mini kit (Qiagen GmBH, Hilden, Germany), according to the manufacturer's protocol. An Applied Biosystems™ High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a miScript II RT kit (Qiagen GmBH) were used for reverse transcription. qScript One-Step RT-qPCR kit (Quanta Bisciences, Inc., Beverly, MA, USA) and mirVana qRT-PCR miRNA Detection kit (Thermo Fisher Scientific, Inc.) were used to perform qPCR reactions in an ABI 7500 System (Applied Biosystems; Thermo Fisher Scientific Inc.). The miRNA-21 primer pair was purchased from Sigma-Aldrich (cat. no. MIRAP00047; Merck KGaA, Darmstadt, Germany). The following primer pairs and the miRNA-21 primers were used for the qPCR: lncRNA-NEF forward, 5'-CTGGCCGCTTAACCAACCC-3' and reverse, 5'-GCCCAAAACAGCTCCTCAATTT-3'; β-actin forward, 5'-GACCTCTATGGCAACACAAGT-3' and reverse, 5'-AGTACTGTGCGTACAGGAGGA-3'; U6 forward, 5'-TCGCTTCGCGACGACA-3' and reverse, 5'-AACGCTTCACGAAATTTGCAGT-3'. The aforementioned primers and vectors were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 5 min, 40 cycles of 95°C for 5 sec and 60°C for 40 sec. mRNA levels were quantified using the 2ΔΔCq method (14). miRNA-21 expression was normalized to U6, while lncRNA-NEF was normalized to β-actin.

Vectors, miRNAs and cell transfection. MISSION® microRNA Mimic hsa-miR-21* (cat no. HM10372) and MISSION® miRNA, Negative Control 1 (cat no. HM0002) were purchased from Sigma-Aldrich; Merck KGaA. lncRNA-NEF expression pRES vectors and empty pRES vectors were purchased from Shanghai GenePharma Co., Ltd. lncRNA-NEF sequence refers to a previous study (13). Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect 5x10⁵ cells with miRNA (50 mM) and vectors (15 mM). Transfection was performed according to the manufacturer’s protocol. Untransfected cells were used as control (C) cells. Cells transfected with MISSION® miRNA, Negative Control 1 or empty vectors were used as negative control (NC) cells. Transfection efficacy was determined by RT-qPCR. Cells were harvested 24 h following transfection for subsequent experimentation.

Transwell migration and invasion assay. Cell migration and invasion were quantified when lncRNA-NEF and miRNA overexpression rates reached 200-250% following 24 h transfection. Cultrex® 96-well cell migration/invasion chambers (8 μm) were purchased from Bio-Technne (Minneapolis, MN, USA). The assays were performed according to manufacturer's protocol. Briefly, serum-free Eagle’s Minimum Essential Medium was used to prepare single cell suspensions (5x10⁴ cells/ml). A total of 5x10³ cells in 100 μl were transferred to the upper chamber of each well, and the lower chamber was filled with Eagle's Minimum Essential Medium supplemented with 20% FBS. Cells were maintained in an incubator (37°C, 5% CO₂) for 2 h. Membranes were subsequently stained with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) at 25°C for 20 min. The invasion assay was performed in the same way as the migration assay; however, the upper chamber was pre-coated with Matrigel (cat no. 356234; Millipore; Merck KGaA) at room temperature for 6 h prior to experimentation.

Statistical analysis. All experiments were repeated three times and data are presented as the mean ± standard deviation. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). Correlations between plasma lncRNA-NEF and miRNA-21 were analyzed by Pearson’s correlation test. Receiver operating characteristic (ROC) curve analysis was performed using patients with osteosarcoma as true positive cases and healthy controls as true negative cases. Comparisons between two groups were performed using the Student's t-test and comparisons among multiple groups were performed by a one-way analysis of variance followed by the Tukey test. P<0.05 was considered to indicate a statistically significant difference.

Results

Plasma levels of lncRNA-NEF and miRNA-21 are altered in patients with osteosarcoma compared with healthy controls. RT-qPCR results revealed that, compared with the healthy control group, plasma levels of lncRNA-NEF were significantly decreased in patients with osteosarcoma (P<0.05; Fig. 1A). By contrast, plasma levels of miRNA-21 were significantly increased in patients with osteosarcoma compared with healthy controls (P<0.05; Fig. 1B). These data suggest that lncRNA-NEF and miRNA-21 may be implicated in osteosarcoma.

Altered plasma levels of lncRNA-NEF and miRNA-21 distinguish patients with osteosarcoma from healthy controls. To evaluate the diagnostic value of lncRNA-NEF and miRNA-21 for osteosarcoma, ROC curve analysis was
performed with patients with osteosarcoma as true positive cases and healthy controls as true negative cases. For plasma IncRNA-NEF, the area under the curve (AUC) was 0.9176, with a standard error of 0.02793 and 95% confidence interval (CI) of 0.8629-0.9724 (Fig. 2A). For plasma miRNA-21, the AUC was 0.9009, with a standard error of 0.02863 and 95% CI of 0.8457-0.9662 (Fig. 2B).

**Plasma IncRNA-NEF and miRNA-21 are significantly and reversely correlated in both patients with osteosarcoma and healthy controls.** Correlations between plasma IncRNA-NEF and miRNA-21 in patients with osteosarcoma and healthy controls were analyzed by the Pearson's correlation test. Results revealed that plasma levels of IncRNA-NEF and miRNA-21 were significantly and reversely correlated in patients with osteosarcoma (P<0.05; Fig. 3A). In addition, a significantly reverse correlation between plasma levels of IncRNA-NEF and miRNA-21 was demonstrated in healthy controls (P<0.05; Fig. 3B).

**IncRNA-NEF overexpression leads to inhibited miRNA-21 expression in two human osteosarcoma cell lines.** The reverse correlation between plasma IncRNA-NEF and miRNA-21 suggests the possibility of interactions between them. To further explore these potential interactions, IncRNA-NEF expression vectors and miRNA-21 mimics were transfected into the human osteosarcoma cell lines MG-63 and U2OS. IncRNA-NEF and miRNA-21 expression was subsequently detected using RT-qPCR (Fig. 4). Compared with the C and NC groups, IncRNA-NEF overexpression significantly decreased expression of miRNA-21 in the two cell lines (P<0.05; Fig. 4A). By contrast, miRNA-21 overexpression resulted in no significant effects on the expression of IncRNA-NEF (P<0.05; Fig. 4B).

**IncRNA-NEF overexpression inhibits migration of osteosarcoma cells, possibly through miRNA-21.** Transwell migration and invasion assays were performed to investigate the effects of IncRNA-NEF and miRNA-21 overexpression on the migration and invasion abilities of MG-63 and U2OS human osteosarcoma cell lines. Compared with the C, control miRNA and empty vector groups, IncRNA-NEF overexpression significantly inhibited and miRNA-21 overexpression significantly promoted migration (P<0.05; Fig. 5A) and invasion (P<0.05; Fig. 5B) of MG-63 and U2OS human osteosarcoma cell lines.
Compared with cells with lncRNA-NEF overexpression alone, cells with lncRNA-NEF and miRNA-21 overexpression exhibited significantly reduced migration (P<0.05; Fig. 5A) and invasion (P<0.05; Fig. 5B) abilities.

**Discussion**

IncRNA-NEF is an oncogenic IncRNA which may inhibit epithelial to mesenchymal transition in hepatocellular carcinoma (13); however, its involvement in other human diseases is unknown. The present study revealed that IncRNA-NEF may inhibit cancer cell migration and invasion in osteosarcoma. These effects may be mediated by the downregulation of miRNA-21, a well-characterized oncogenic miRNA in cancer biology (11).

Treatment of osteosarcoma is challenged by the high prevalence rate of tumor metastasis by the time of diagnosis (2). Therefore, early diagnosis is required to improve patient outcomes. The current study enrolled patients with stage I and II osteosarcoma, which are early stages of osteosarcoma. Downregulation of IncRNA-NEF was previously observed in patients with hepatocellular carcinoma (13). The present study revealed significantly decreased plasma levels of IncRNA-NEF in patients with osteosarcoma compared with healthy controls. Downregulation of plasma IncRNA-NEF effectively distinguished patients with osteosarcoma from...
healthy controls, indicating that decreased levels of plasma lncRNA-NEF may have diagnostic value in the early detection of osteosarcoma. Subsequent in vitro experiments revealed that lncRNA-NEF may promote cancer cell invasion and migration in osteosarcoma. Therefore, the downregulation of lncRNA-NEF in patients with osteosarcoma may be used to predict tumor metastasis. lncRNA-NEF may serve as a therapeutic target for the prevention of tumor metastasis in osteosarcoma.

As an oncogenic miRNA, miRNA-21 is upregulated in different types of human malignancies, including osteosarcoma (15-17). Consistent with previous studies, the current study observed significantly upregulated plasma miRNA-21 in patients with osteosarcoma compared with healthy controls. Overexpression of miRNA-21 distinguished patients with osteosarcoma from healthy controls. However, altered expression of miRNA-21 has been observed in different types of cancer (11,15-17). Therefore, the diagnostic specificity of plasma miRNA-21 for osteosarcoma may be low.

The present study demonstrated a reverse correlation between plasma miRNA-21 and lncRNA-NEF in both patients with osteosarcoma and healthy controls. In vitro experiments using osteosarcoma cell lines revealed that lncRNA-NEF is a potential upstream inhibitor of miRNA-21 in the regulation of migration and invasion of osteosarcoma cells. lncRNA-NEF overexpression failed to significantly affect osteosarcoma cell proliferation (data not shown, revealed by Cell Counting Kit-8 assay). miRNA-21 has been previously reported to be involved in the regulation of cancer proliferation in osteosarcoma (17-19). Therefore, lncRNA-NEF may antagonize the effects of downregulation of miRNA-21 on cancer cell proliferation. The current study reported that lncRNA-NEF may interact with a miRNA to participate in cancer development.

In conclusion, lncRNA-NEF is downregulated and miRNA is upregulated in patients with osteosarcoma compared with healthy controls. lncRNA-NEF may inhibit cancer cell migration and invasion in osteosarcoma by downregulating miRNA-21.
Acknowledgements
Not applicable.

Funding
No funding was received.

Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
QLY, HY, XH, QSY and CZ performed the experiments. QLY analyzed the data and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Ethical approval was obtained from the Ethics Committee of Guangzhou General Hospital of Guangzhou Military Command. The study followed the tenets of the Declaration of Helsinki, and written informed consent was obtained from all patients and controls after the nature and possible consequences of the study were explained.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References
1. Ottaviani G and Jaffe N: The epidemiology of osteosarcoma. Cancer Treat Res 152: 3-13, 2009.
2. Marko TA, Diessner BJ and Spector LG: Prevalence of metastasis at diagnosis of osteosarcoma: An international comparison. Pediatr Blood Cancer 63: 1006-1011, 2016.
3. Luetke A, Meyers PA, Lewis I and Juergens H: Osteosarcoma treatment—where do we stand? A state of the art review. Cancer Treat Rev 40: 523-532, 2014.
4. Li JZ, Tian ZQ, Jiang SN and Feng T: Effect of variation of ABCB1 and GSTP1 on osteosarcoma survival after chemotherapy. Genet Mol Res 13: 3186-3192, 2014.
5. Anderson ME: Update on survival in osteosarcoma. Orthop Clin North Am 47: 283-292, 2016.
6. Denduluri SK, Wang Z, Yan Z, Wang J, Wei Q, Mohammed MK, Haydon RC, Lui H and He TC: Molecular pathogenesis and therapeutic strategies of human osteosarcoma. J Biomed Res 30: 2015.
7. Mattick JS and Makunin IV: Non-coding RNA. Hum Mol Genet 15: R17-R29, 2006.
8. Eddy SR: Non-coding RNA genes and the modern RNA world. Nat Rev Genet 2: 919-929, 2001.
9. Esteller M: Non-coding RNAs in human disease. Nat Rev Genet 12: 861-874, 2011.
10. Taft RJ, Pang KC, Mercer TR, Dinger M and Mattick JS: Non-coding RNAs: Regulators of disease. J Pathol 220: 126-139, 2010.
11. Kumarswamy R, Volkmann I and Thum T: Regulation and function of miRNA-21 in health and disease. RNA Biol 8: 706-713, 2011.
12. Dan J, Wang J, Wang Y, Zhu M, Yang X, Peng Z, Jiang H, and Chen L: LncRNA-MEG3 inhibits proliferation and metastasis by regulating miRNA-21 in gastric cancer. Biomed Pharmacother 99: 931-938, 2018.
13. Liang WC, Ren JL, Song CW, Chen YP, et al: Dysregulation of miRNA-21 and their potential as biomarkers for the diagnosis of cervical cancer. Int J Clin Exp Pathol 8: 7131-7139, 2015.
14. Han Y, Xu GX, Lu H, Yu DH, Ren Y, Wang L, Huang XH, Hou WJ, Wei ZH, Chen YP, et al: MiRNA-21 silencing mediated by tumor-targeted nanoparticles combined with sunitinib: A new multimodal gene therapy approach for glioblastoma. J Control Release 207: 31-39, 2015.
15. Zhang QL, Yang SH, Liu HY and Wu CH: Inhibition of osteosarcoma cell proliferation by a short hairpin RNA targeting proliferation cell nuclear antigen. Zhonghua Bing Li Xue Za Zhi 34: 167-170, 2005 (In Chinese).
16. Li C, Xu B, Miu X, Deng Z, Liao H and Hao L: Inhibition of miRNA-21 attenuates the proliferation and metastasis of human osteosarcoma by upregulating PTEN. Exp Ther Med 15: 1036-1040, 2018.
17. Zhao J, Zhang C, Gao Z, Wu H, Gu R and Jiang R: Long non-coding RNA ASBEL promotes osteosarcoma cell proliferation, migration, and invasion by regulating microRNA-21. J Cell Biochem 119: 6461-6469, 2018.