lncRNA NEF inhibits glioma by downregulating TGF-β1

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Abstract. NEF is a tumor suppressing long non-coding (lnc)RNA in hepatocellular carcinoma. Based on current literature, the involvement of NEF in other human diseases is still unknown. The current study aimed to investigate the potential involvement of NEF in glioma, which is a type of rare, but aggressive malignancy. It was determined that NEF expression was downregulated in tumor tissues compared with adjacent heathy tissues. A low blood NEF level in patients with glioma effectively distinguished patients from healthy controls who had high blood NEF levels. Blood NEF levels were significantly correlation with distant tumor metastasis, but not tumor growth. Blood NEF levels were negatively correlated with blood transforming growth factor (TGF)-β1 levels in patients with distant tumor metastasis, but not in patients with non-metastatic glioma and healthy controls. NEF overexpression inhibited cancer cell migration and invasion. In addition, NEF overexpression downregulated TGF-β1 expression. The authors of the current study concluded that lncRNA NEF may inhibit glioma cell migration and invasion by downregulating TGF-β1.

Introduction

Tumor metastasis is the main cause of mortality among patients with different types of cancers (1). Glioma as one of the main type of central nervous system tumor accounts for more than 80% cases of all malignant brain tumors (2). Glioma causes a series of clinical symptoms, including vomiting, cranial nerve disorders, seizures, headaches and loss of vision (3,4). However, these clinical symptoms can easily be ignored or misdiagnosed. Therefore, the existence of distant tumor metastasis is common by the time of diagnosis (5). Investigating how to prevent and treat glioma metastasis is a major task for clinical researchers as the efficacy of treatment is usually poor due to the unknown pathogenesis of glioma (3-5).

As a double edged sword in cancer biology, transforming growth factor (TGF)-β signaling inhibits tumor growth at the early stage of tumor development, but promotes tumor metastasis in late stages (6). TGF-β signaling also has been demonstrated to enhance the invasion and metastasis of glioma, and the inhibition of TGF-β signaling has been considered to be a therapeutic target for glioma (7). Successful TGF-β signaling transduction requires the involvement of different long non-coding (lnc)RNAs (8,9), which are a subgroup of non-coding RNAs composed of more than 200 nucleotides and have critical functions in cancer biology (10). NEF is a novel lncRNA and has been determined to have a tumor suppressive role in hepatocellular carcinoma (11). In the current study, the authors hypothesized that lncRNA NEF may inhibit glioma cell migration and invasion by downregulating TGF-β1.

Materials and methods

Patients and specimens. A total of 53 patients with glioma were selected in Yichang Second People's Hospital (Yichang, China) between March 2015 and January 2018. Inclusion criteria: i) Patients pathologically diagnosed with glioma and treated for the first time; ii) patients willing to participate. Exclusion criteria: i) Patients complicated with other severe diseases; ii) patients who received treatment prior to admission. These patients included 30 males and 23 females; the age ranged was from 29 to 69 years, with a mean age of 49.1±5.3 years. At the same time, a total of 56 healthy controls were also included to serve as control group. The control group included 31 males and 25 females; the age ranged was from 28 to 68 years, with a mean age of 48.8±6.1 years. Biopsies of tumor tissues (100-200 mg) and adjacent heathy tissues (100-200 mg) were obtained from each patient with glioma. Blood samples (5 ml) were collected from patients with glioma as well as participants in the control group on the day of admission. No significant differences in gender, age and other basic clinical data between the patient group and the control group were identified (Table I). The ethics committee of Yichang Second People's Hospital approved the current study. All participants signed informed consent forms.

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Cell culture. Hs 683 (ATCC® HTB-138™) and CCD-25Lu (ATCC® CCL-215™) human glioma cell lines were bought from American Type Culture Collection (Manassas, VA, USA). Cells were culture in Eagle's Minimum Essential Medium (cat. no. 30-2003™; American Type Culture Collection) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany and maintained at 37˚C in a 5% CO_2 humidified incubator.

In cases of TGF-β1 treatment, cells were pre-treated with 5, 10, 20 and 30 ng/ml TGF-β1 in culture medium for 24 h prior to subsequent experimentation.

Cell transfection. Full-length cDNA for NEF was PCR amplified introducing *EcoRI* restriction sites and cloned into the pIRSE2 vector (Clontech Laboratories, Inc., Mountainview, CA, USA) by Sangon Biotech Co., Ltd. (Shanghai, China). pIRSE2-NEF vectors (10 nM) were mixed with Lipofectamine® 2000 reagent (cat. no. 11668-019; Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) to form vector-reagent complexes and used to transfect cells. Cells ($1x10^5$ cells in each well of a six-well plate) were subsequently transfected with vector-reagent complexes at 37˚C for 5 h. Cells without transfection were used as control cells. Cells transfected with empty vectors were used as negative control cells. Transfection efficiency >200% compared with control cells was achieved in each experiment.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells and blood samples using TRIzol® reagent (Invitrogen, Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Inc.) using the following thermocycling conditions: 25˚C for 5 min, 50˚C for 20 min and 75˚C for 5 min. qPCR was subsequently performed using the SYBR® Green Real-Time PCR Master mix (Thermo Fisher Scientific, Inc.). The following primer pairs were used for the qPCR: lncRNA-NEF forward, 5'-CTG CCGTCTTAAACCAACCC-3' and reverse, 5'-GCCCAAACA GCTCCTCAATT-3'; β-actin forward, 5'-GACCTCTATGCC AACACAGT-3' and reverse, 5'-AGTACTTGCGCTCAGGAG GA-3'. The following thermocycling conditions were used: Initial denaturation at 95˚C for 42 sec; 40 cycles of 95˚C for 22 sec and 56.5˚C for 38 sec. lncRNA-NEF levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to β-actin as the internal control (12).

ELISA. An ELISA kit was used to measure blood levels of TGF-β1 (cat. no. RAB0460-1KT; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol.

In vitro cell migration and invasion assays. Transwell migration and invasion assays were performed to examine *in vitro* cell migration and invasion. Following transfection, Hs 683 and CCD-25Lu cell suspensions with a density of $5x10^4$ cells/ml were made. In the migration assay, $5x10^3$ cells in 0.1 ml serum-free Eagle's Minimum Essential Medium were seeded into the upper chamber, while the lower chamber was filled with RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 20% fetal calf serum (Sigma-Aldrich; Merck KGaA). Membranes were collected after cell culture was performed for 24 h in an incubator at 37˚C with 5% CO_2. Membranes were stained with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) for 30 min at 25˚C. Cells were counted under an optical microscope (magnification, 40x; CX33, Olympus Corporation, Tokyo, Japan). The cell invasion assay was performed using the same protocol, however the upper chambers were precoated with Matrigel (cat. no. 356234; EMD Millipore, Billerica, MA, USA).

Western blot analysis. Following transfection, total protein was extracted from Hs 683 and CCD-25Lu cells using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total protein was quantified using a bicinchoninic acid assay and 30 μg protein/well was separated using SDS-PAGE on a 12% gel. The separated proteins were transferred onto polyvinylidene difluoride membranes and blocked for 2 h with 5% skimmed milk. The membranes were incubated with primary antibodies against TGF-β1 (cat. no. ab92486) and GAPDH (cat. no. ab9485; both 1:1,000; Abcam) overnight at 4˚C. Following primary incubation, membranes were further incubated with anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies (1:1,000; cat. no. MBS435036; MyBioSource) for 2 h at room temperature. Protein bands were visualized using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.) and signals were detected using MYECL™ Imager (Thermo Fisher Scientific, Inc.). Protein expression was quantified using ImageJ software (version 1.6; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Graphpad Prism 6 software was used for all data analysis. Gene expression, and cell migration and invasion data were recorded as mean ± standard deviation. Data were compared by t-test (between two groups) or one-way analysis of variance followed by a Fisher's Least Significant Difference test (among multiple groups). Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic value of blood NEF for glioma. Pearson's correlation coefficient was used to analyze the correlations between blood NEF and TGF-β1 expression levels. Chi-square test was performed to analyze the associations between blood NEF expression and clinicopathological data of patients. $P<0.05$ was considered to be statistically significant.

Results

NEF expression is lower in tumor tissues compared with adjacent healthy tissues in the majority of patients with glioma. Differential expression of a gene in tumor tissues and adjacent healthy tissues indicated its involvement in cancers. Therefore, the expression of NEF in tumor tissues and adjacent healthy tissues of 53 patients with glioma was detected. As presented in Fig. 1, significantly lower expression levels of NEF in tumor tissues was identified in 47 out of 53 patients when compared with adjacent healthy tissues, accounting for 88.7% of the patients. Therefore, the downregulation of lncRNA NEF is likely involved in the pathogenesis of glioma.

Table I. Associations between the expression of NEF in the blood and clinicopathological data of patients with glioma.

Characteristic	Groups	Cases	Number of patients		χ^2	P-value
			High-expression	Low-expression		
Age, years	>50	28	16	12	0.91	0.34
	≤50	25	11	14		
Sex	Male	30	14	16	0.51	0.48
	Female	23	13	10		
Primary tumor diameter, cm	>2	26	15	11	0.93	0.33
	≤2	27	12	15		
Tumor distant metastasis	Yes	31	12	19	4.47	0.03
	No	22	15	7		

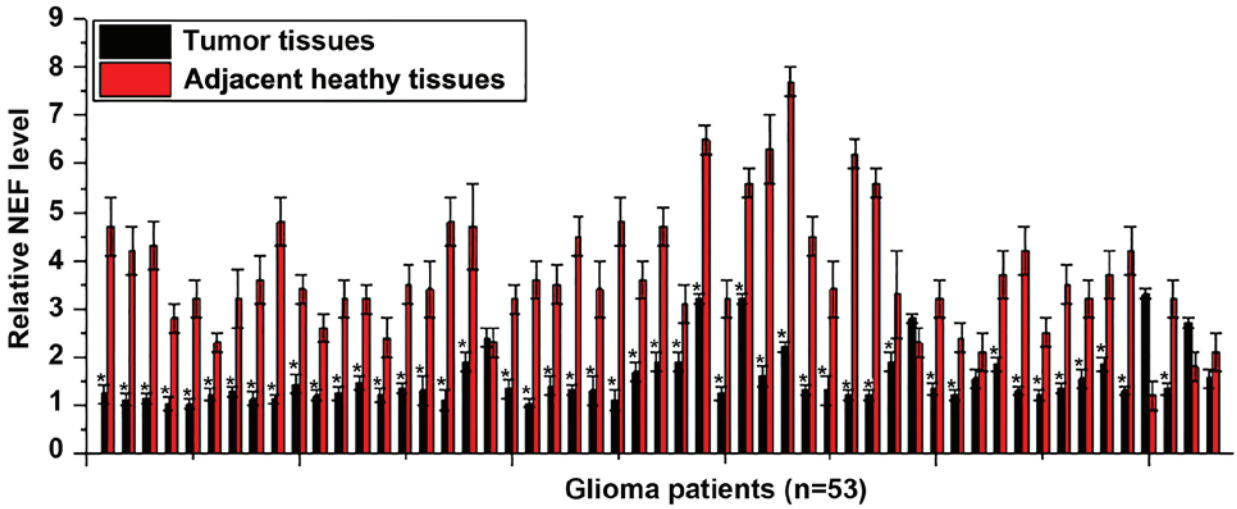

Figure 1. NEF expression is lower in tumor tissues compared with adjacent healthy tissues in the majority of patients with glioma. Relative NEF expression in tumor tissues and adjacent heathy tissues patients with glioma. *P<0.05 vs. adjacent healthy tissues.

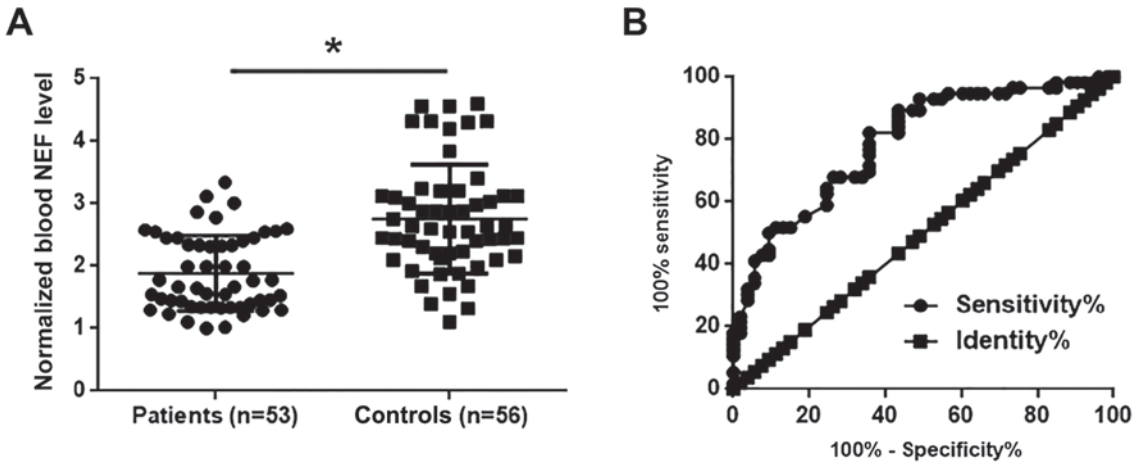

Figure 2. Low blood levels of NEF in glioma patients effectively distinguishes patients from healthy controls. (A) Blood levels of NEF in glioma patients and healthy controls. (B) A receiver operating characteristic curve of the diagnosis of glioma using blood NEF. *P<0.05.

Low blood levels of NEF in glioma patients effectively distinguishes patients from healthy controls. Blood levels of NEF in glioma patients and healthy controls were measured by RT-qPCR. As presented in Fig. 2A, blood levels of NEF were significantly lower in patients with glioma compared with healthy controls (P<0.05). ROC curve analysis was performed

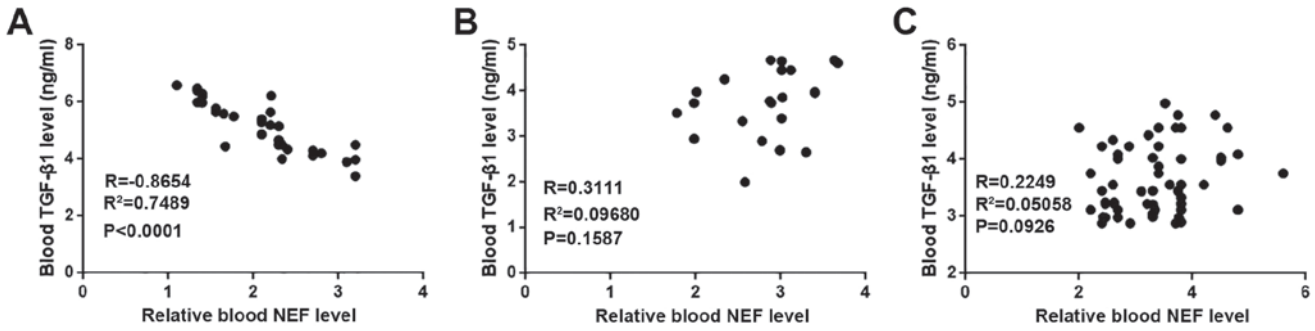

Figure 3. Blood levels of NEF are significantly correlated with TGF-β1 in patients with metastatic glioma. Pearson's correlation coefficient analysis of correlations between blood NEF and TGF-β1 levels in (A) patients with metastatic glioma, (B) patients with non-metastatic glioma and (C) healthy controls. TGF, transforming growth factor.

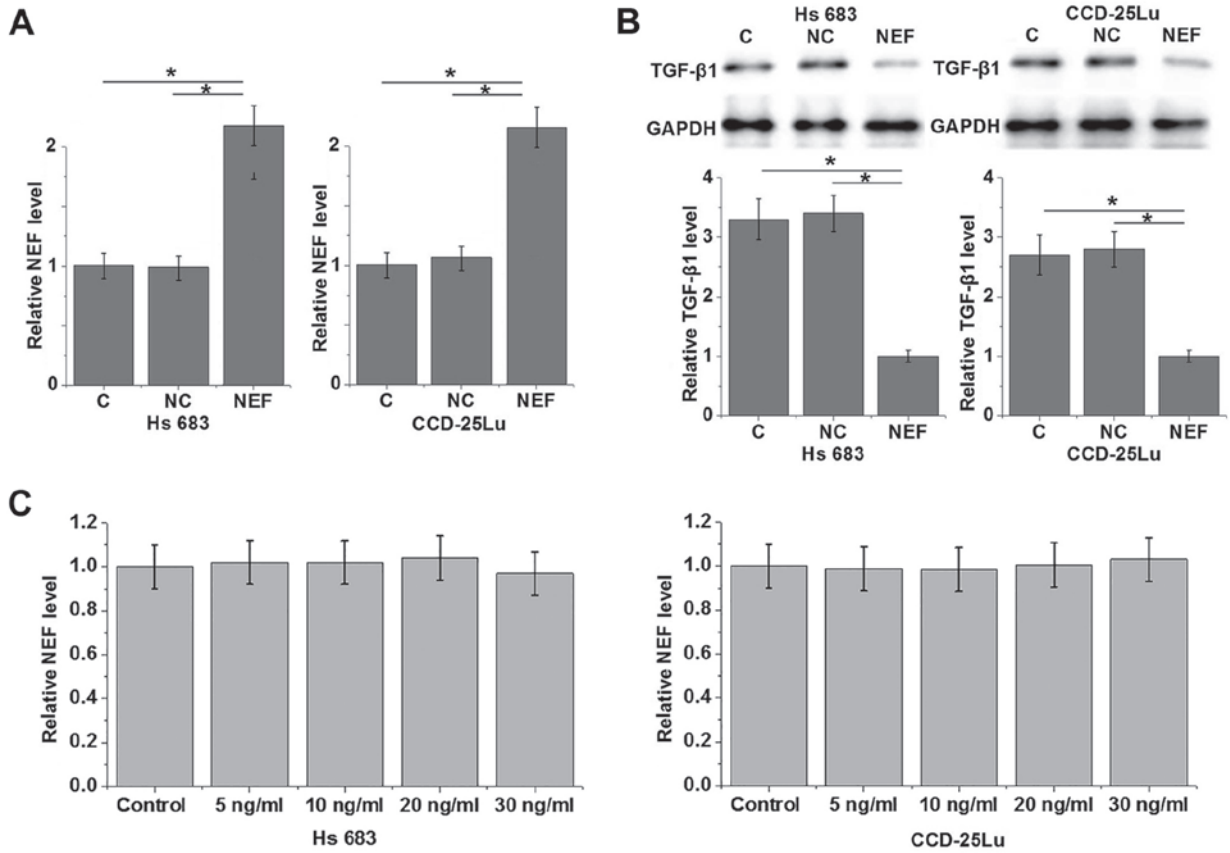

Figure 4. NEF is likely an upstream inhibitor of TGF-β1 in glioma cells. (A) NEF expression and (B) TGF-β1 expression in non-transfected cells, cells transfected with empty vectors and cells transfected with NEF overexpressing vectors from two human glioma cell lines. (C) NEF expression in Hs 683 cells following treatment with 5, 10, 20 and 30 ng/ml TGF-β1. C, control; NC, negative control; TGF, transforming growth factor. *P<0.05.

to evaluate the diagnostic value of blood NEF for glioma. As presented in Fig. 2B, area under the curve was 0.7908, with standard error of 0.04271 and 95% confident interval of 0.7070-0.8745.

Blood levels of NEF significantly associate with distant tumor metastasis. Patients were divided into high (n=27) and low (n=26) expression groups based on NEF expression cut-off score of 2.03. A chi-square test was performed to analyze the association between the blood NEF levels and clinicopathological data of patients with glioma. No significant associations were identified between NEF blood levels, and patients' age, sex and tumor size (Table I). By contrast, a significant association between blood levels of NEF and the existence of distant tumor metastasis was observed.

Blood levels of NEF are significantly correlated with TGF-β1 in patients with metastatic glioma. Pearson's correlation coefficient was used to analyze the correlations between blood NEF and TGF-β1 levels. Blood levels of NEF were significantly correlated with TGF-β1 in patients with metastatic glioma (Fig. 3A). In contrast, no significant correlations between blood NEF and TGF-β1 were identified in patients with non-metastatic glioma (Fig. 3B) and healthy controls (Fig. 3C).

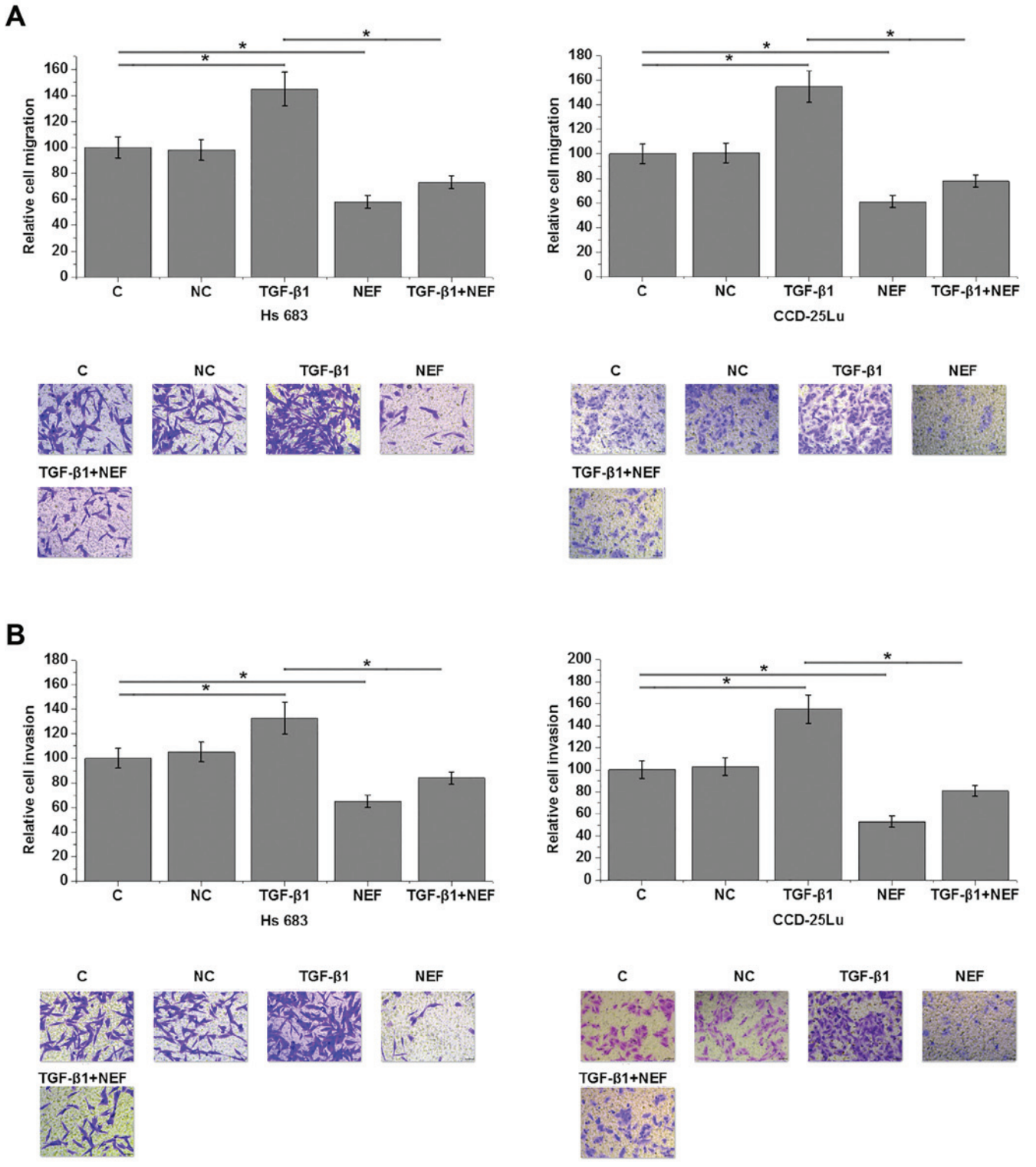

Figure 5. NEF overexpression promotes the migration and invasion of glioma cells. (A) Migration and (B) invasion ability of non-transfected cells, cells transfected with empty vectors, NEF overexpressing vectors or exogenous TGF-β1, and cells co-transfected with NEF overexpressing vectors and exogenous TGF-β1 from two human glioma cell lines. C, control; NC, negative control; TGF, transforming growth factor. *P<0.05.

NEF is likely an upstream inhibitor of TGF-β1 in glioma cells. To further investigate the interactions between NEF and TGF-β1, NEF expression vectors were transfected into Hs 683 and CCD-25Lu cells. RT-qPCR revealed that the expression of NEF was significantly upregulated in both cell lines following transfection compared with control and negative control groups (Fig. 4A; P<0.05). Compared with control and negative control cells, cells with NEF overexpression demonstrated significantly downregulated TGF-β1 protein expression (all P<0.05; Fig. 4B). By contrast, treatment with exogenous TGF-β1 at concentrations of 5, 10, 20 and 30 ng/ml demonstrated no significant effects on NEF expression (Fig. 4C). Therefore NEF is likely an upstream inhibitor of TGF-β1 in glioma cells.

NEF overexpression promotes the migration and invasion of glioma cells. Cell migration and invasion data demonstrated that, compared with control, cells with NEF overexpression significantly inhibited cell migration (all P<0.05; Fig. 5A) and invasion (all P<0.05; Fig. 5B). In addition, treatment with exogenous TGF-β1 at a dosage of 10 ng/ml significantly promoted cell migration (all P<0.05; Fig. 5A) and invasion (all P<0.05; Fig. 5B) compared with control cells. Exogenous TGF-β1 treatment also significantly reduced the inhibitory effects of NEF overexpression on cell migration (all P<0.05; Fig. 5A) and invasion (all P<0.05; Fig. 5B) compared with cells treated with exogenous TGF-β1 alone.

Discussion

lncRNA NEF suppresses epithelial to mesenchymal transition (EMT) in hepatocellular carcinoma, which in turn inhibits tumor invasion and metastasis (11). The current study suggests that lncRNA NEF also serves a tumor suppression role in glioma by inhibiting glioma cell migration and invasion. The actions of lncRNA NEF may be mediated by the activation of the TGF-β signaling pathway, which is critical for EMT in a variety of types of cancers (12).

The development of glioma altered the expression of a large set of lncRNAs and the expression pattern of differentially expressed lncRNAs has been demonstrated to determine the clinical phenotype of glioma (13). Upregulation of lncRNA MALAT1 was observed in patients with glioma and the overexpression of this lncRNA at least partially contributed to the malignant nature of this disease (14). By contrast, lncRNA TUG1 is downregulated in human glioma and the overexpression of TUG1 promoted cancer cell apoptosis, indicating the potential application of lncRNA TUG1 as a therapeutic target for glioma (15). In the current study, the authors examined the expression of lncRNA NEF in the tumor tissues and adjacent heathy tissues of glioma patients, and in the blood of patients and healthy controls due to difficulties in collecting brain biopsies from healthy controls. It was observed that NEF was downregulated in tumor tissues compared with adjacent heathy tissues and that blood levels of NEF in patients with glioma were lower compared with healthy controls, indicating the potential role of lncRNA NEF in glioma.

Early diagnosis is critical for the survival of patients with glioma as a considerable proportion of patients are diagnosed following distant tumor metastasis (5). With the advantages of less invasive techniques, circulating biomarkers have been increasing used in the clinical diagnosis of human diseases, such as glioma (16). In contrast to other cancers, glioma cells migrate through cerebrospinal fluid (17). In the current study, blood was used instead of cerebrospinal fluid to detect circulating NEF due to difficulties in enrolling enough volunteers willing to donate cerebrospinal fluid. However, blood biomarkers have also been revealed to have value in diagnosing glioma (18). The current study indicated that low blood levels of lncRNA NEF can be used to effectively distinguish glioma patients from healthy controls. Therefore, NEF may be used to improve the diagnosis of glioma.

The current study observed a significant association between blood levels of NEF and the existence of distant tumor metastasis in patients with glioma, indicating the potential involvement of NEF in glioma. TGF-β signaling is a key regulator in the invasion and metastasis of glioma cells (7). The authors of the current study observed a significant negative correlation between blood NEF and TGF-β1 in patients with metastatic glioma, but not in patients with non-metastatic glioma and healthy controls. Also, TGF-β1 treatment did not affect NEF expression, suggesting that NEF is upstream of TGF-β1 in the metastasis of glioma. TGF-β signaling has been demonstrated to be activated in the growth and metastasis of glioma (7). *In vitro* cell experiment data also supported this hypothesis through the following observations: i) NEF overexpression promoted TGF-β1 expression; ii) exogenous TGF-β1 did not have an effect on NEF expression; iii) NEF overexpression inhibited and exogenous TGF-β1 promoted the migration and invasion of glioma cells; iv) exogenous TGF-β1 treatment reduced the inhibitory effects of NEF overexpression on cell migration and invasion. Therefore, NEF may inhibit glioma metastasis by inactivating TGF-β signaling.

Due to the low incidence rate of glioma, the sample size in the current study is relative low. Future studies will try to enroll more patients to further confirm the conclusions of the current study. In conclusion, lncRNA NEF was downregulated in glioma and NEF overexpression may inhibit the metastasis of glioma by serving as an upstream inhibitor of the TGF-β signaling pathway.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

QH and HC designed the experiments. QH, HC, BZ and CC performed the experiments. WY and YY analyzed the data. HC prepared the manuscript. All other authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Yichang Second People's Hospital (Yichang, China). All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Mehlen P and Puisieux A: Metastasis: A question of life or death. Nat Rev Cancer 6: 449-458, 2006.
2. Valastyan S and Weinberg RA: Tumor metastasis: Molecular insights and evolving paradigms. Cell 147: 275-292, 2011.
3. Omuro A and DeAngelis LM: Glioblastoma and other malignant gliomas: A clinical review. JAMA 310: 1842-1850, 2013.
4. Mishra MV, Andrews DW, Glass J, Evans JJ, Dicker AP, Shen X and Lawrence YR: Characterization and outcomes of optic nerve gliomas: A population-based analysis. J Neurooncol 107: 591-597, 2012.
5. Stark AM, van de Bergh J, Hedderich J, Mehdorn HM and Nabavi A: Glioblastoma: Clinical characteristics, prognostic factors and survival in 492 patients. Clin Neurol Neurosurg 114: 840-845, 2012.
6. Akhurst RJ and Derynck R: TGF-beta signaling in cancer-a double-edged sword. Trends Cell Biol 11: S44-S51, 2001.
7. Han J, Alvarez-Breckenridge CA, Wang QE and Yu J: TGF-β signaling and its targeting for glioma treatment. Am J Cancer Res 5: 945-955, 2015.
8. Yuan J, Yang F, Wang F, Ma JZ, Guo YJ, Tao QF, Liu F, Pan W, Wang TT, Zhou CC, *et al*: A long noncoding RNA activated by TGF-β promotes the invasion-metastasis cascade in hepatocellular carcinoma. Cancer Cell 25: 666-681, 2014.
9. Fan Y, Shen B, Tan M, Mu X, Qin Y, Zhang F and Liu Y: TGF-β-induced upregulation of malat1 promotes bladder cancer metastasis by associating with suz12. Clin Cancer Res 20: 1531-1541, 2014.
10. Prensner JR and Chinnaiyan AM: The emergence of lncRNAs in cancer biology. Cancer Discov 1: 391-407, 2011.
11. Liang WC, Ren JL, Wong CW, Chan SO, Waye MM, Fu WM and Zhang JF: LncRNA-NEF antagonized epithelial to mesenchymal transition and cancer metastasis via cis-regulating FOXA2 and inactivating Wnt/β-catenin signaling. Oncogene 37: 1445-1456, 2018.
12. Zhang J, Tian XJ and Xing J: Signal transduction pathways of EMT induced by TGF-β, SHH, and WNT and their crosstalks. J Clin Med 5: E41, 2016.
13. Zhang X, Sun S, Pu JK, Tsang AC, Lee D, Man VO, Lui WM, Wong ST and Leung GK: Long non-coding RNA expression profiles predict clinical phenotypes in glioma. Neurobiol Dis 48: 1-8, 2012.
14. Ma KX, Wang HJ, Li XR, Li T, Su G, Yang P and Wu JW: Long noncoding RNA MALAT1 associates with the malignant status and poor prognosis in glioma. Tumor Biol 36: 3355-3359, 2015.
15. Li J, Zhang M, An G and Ma Q: LncRNA TUG1 acts as a tumor suppressor in human glioma by promoting cell apoptosis. Exp Biol Med (Maywood) 241: 644-649, 2016.
16. Kros JM, Mustafa DM, Dekker LJ, Sillevis Smitt PA, Luider TM and Zheng PP: Circulating glioma biomarkers. Neuro Oncol 17: 343-360, 2015.
17. Pan W, Gu W, Nagpal S, Gephart MH and Quake SR: Brain tumor mutations detected in cerebral spinal fluid. Clin Chem 61: 514-522, 2015.
18. Westphal M and Lamszus K: Circulating biomarkers for gliomas. Nat Rev Neurol 11: 556-566, 2015.