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LncRNA-NEF inhibits proliferation, migration and invasion of esophageal squamous-cell carcinoma cells by inactivating wnt/β-catenin pathway

J. ZHANG, S.-L. HU, C.-H. QIAO, J.-F. YE, M. LI, H.-M. MA, J.-H. WANG, S.-Y. XIN, Z.-L. YUAN

Department of Gastroenterology, Xiangyang No. 1 People's Hospital, Hubei University of Medicine, Xiangyang City, Hubei Province, PR. China

Jing Zhang and *Shunlin Hu* contributed equally to this work

Abstract. – OBJECTIVE: Our study aimed to investigate the role of lncRNA-Neighboring Enhancer of FOXA2 (NEF) in esophageal squamous-cell carcinoma.

PATIENTS AND METHODS: Tumor tissues and adjacent tissues were obtained from esophageal squamous-cell carcinoma patients, and blood samples were extracted from both patients with esophageal squamous-cell carcinoma and healthy volunteers. The expression of NEF was detected by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). All patients were followed-up for 5 years and ROC curve analysis and survival analysis were performed to evaluate the diagnostic and prognostic values of serum NEF for esophageal squamous-cell carcinoma. NEF expression vector was transfected into cells of esophageal squamous-cell carcinoma cell lines. Cell proliferation, migration and invasion were detected by CCK-8 assay, transwell migration assay, and transwell invasion assay, respectively. The interaction between NEF and wnt/β-catenin pathway were explored by Western blot and qRT-PCR.

RESULTS: Expression of NEF was significantly downregulated in tumor tissues than in adjacent tissues in most patients. Serum level of NEF was higher in esophageal squamous-cell carcinoma patients than in healthy controls, and was significantly correlated with tumor size and tumor distant tumor metastasis. Serum NEF is a promising diagnostic and prognostic marker for esophageal squamous-cell carcinoma. NEF overexpression inhibited cancer cell proliferation, migration and invasion. NEF overexpression decreased the expression levels of wnt/β-catenin pathway-related proteins, while Wnt activator showed no significant effects on NEF. However, Wnt inhibitor reduced the effects of NEF overexpression on cell proliferation, migration and invasion.

CONCLUSIONS: LncRNA NEF may inhibit the proliferation, migration and invasion of esophageal squamous-cell carcinoma cells by inactivating with wnt/β-catenin pathway.

Key Words:
Esophageal squamous-cell carcinoma, LncRNA NEF, Wnt/β-catenin pathway.

Introduction

Esophageal cancer as one of the least studied and deadliest malignancies is one of the most common types of malignancies in developing countries[1]. World widely, esophageal cancer ranks the 6th place among all causes of cancer-related deaths due to its extremely aggressive nature[2]. China accounts for more than 80% of cases of this disease, and esophageal cancer is considered to be a major public health burden of this country[3]. Esophageal cancer is divided into esophageal adenocarcinoma and esophageal squamous-cell carcinoma, two major primary histologic subtypes[4]. Although the incidence of esophageal squamous-cell carcinoma decreased significantly during the last several decades, it's still the major burden of esophageal cancer[5]. Development of esophageal squamous-cell carcinoma is a complicated process, and the unknown pathogenesis is one of the major causes of failure of treatment[6]. Genetic factors play pivotal roles in the development of esophageal squamous-cell carcinoma. Mutations in tumor suppressor genes and oncogenes such as genes involved in Hippo and Notch pathways play different roles to pro-

Corresponding Author: Zhengli Yuan, MD; e-mail: heqruj1@163.com

mote or inhibit tumor progression[7]. It has been proved[8] that the development of esophageal squamous-cell carcinoma is accompanied by changes in expression of certain lncRNAs, indicating the involvement of lncRNAs in the pathogenesis of this disease. NEF is a recently identified lncRNA which plays a role as tumor suppressor gene in hepatocellular carcinoma, and the action of NEF in hepatocellular carcinoma is proved to be achieved through its interactions with Wnt/β-catenin pathway[9]. In the present work we observed that NEF inhibited proliferation, migration and invasion of esophageal squamous-cell carcinoma cells possibly by inactivating with Wnt/β-catenin pathway.

Patients and Methods

Patients

Our study included 78 patients with esophageal squamous-cell carcinoma who were pathologically diagnosed and treated in Xiangyang No. 1 People's Hospital from January 2011 to January 2013. Exclusion criteria: (1) patients with other types of malignancies; (2) patients with severe coagulation dysfunction; (3) patients with other serve diseases; (4) patients had been treated before admission. Inclusion criteria: (1) patients diagnosed as esophageal squamous-cell carcinoma through pathological examinations; (2) patients diagnosed and treated for the first time; (3) patients who were suitable for surgical resection; (4) patients signed informed consent; (5) patients with complete follow-up data. There were 40 males and 38 females, and age range was 26-72 years, with an average age of 51.1 ± 8.2 years. Our study also included 55 healthy people during the same time period to serve as control group. Control group included 29 males and 26 females, and age range was 25-74 years, with an average age of 50.4 ± 7.7 years. There were no significant differences in age and gender between two groups. This study was approved by the Ethics Committee of Xiangyang No. 1 People's Hospital. All participants signed the informed consent.

Tissue Collection and Serum Preparation

Tumor tissues and adjacent healthy tissues within 2 cm around tumors were collected during surgical resection. About 10 ml of blood were extracted from the elbow vein of each participant on the day of admission. Blood was kept at room temperature for 1.5 hours, followed by centrifugation at room temperature (1000 g) for 20 min. Tissues and serum samples were stored in liquid nitrogen before use.

Cell Lines, Cell Culture and Transfection

Our work included two *Homo sapiens* esophageal squamous-cell carcinoma cell lines KYSE510 and EC9706 (ATCC, Manassas, VA, USA). Cells of KYSE510 and EC9706 cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Invitrogen, Waltham, MA, USA). Cell culture was performed in an incubator (37°C, 5% CO_2). Full-length NEF cDNA fragment with EcoRI cutting site on both ends was obtained through PCR, and was inserted into pIRSE2-EGFP (Clontech, Palo Alto, CA, USA) vector to establish NEF expression vector. Lipofectamine 2000 (11668-019, Invitrogen, Carlsbad, CA, USA) was used to transfect 10 nM NEF expression vector or empty vector (negative control) into 5×10^6 cells. Expression of NEF was checked by qRT-PCR to make sure the upregulation was above 200% before subsequent experiments.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract total RNA. To achieve complete cell lysis, tissues were ground in liquid nitrogen before the addition of TRIzol® reagent. SuperScript IV reverse transcriptase kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to synthesize cDNA. SYBR® Green Real-Time PCR Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to prepare PCR reaction systems. Sequences of primers used in PCR reactions were: 5'-CTGCCGTCTTAAACCAACCC-3' (forward) and 5'-GCCCAAACAGCTCCTCAATT-3' for human lncRNA-NEF; 5'-CCCACTCCTCCACCTTTGAC-3' (forward) and 5'-ATGAGGTCCACCACCCTGTT-3' (reverse) for human GAPDH. PCR reaction conditions were: 95°C for 50 s, followed by 40 cycles of 95°C for 10 s and 55°C for 45 s. Expression level of lncRNA NEF was normalized to endogenous control GAPDH using $2^{-\Delta\Delta Ct}$ method.

Cell Proliferation Assay

Cells of KYSE510 and EC9706 cell lines were collected and cell suspension was prepared with a cell density of 4×10^4 cells/m. Cell suspension

(100 µl, 4×10^3 cells) was transferred to each well of a 96-well plate. Cells were cultured in an incubator (37°C, 5% CO_2), and 10 uL CCK-8 solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA) squamous were added 24, 48, 72 and 96 h later. Cell culture was performed for another 4 h, and OD values at 450 nm were measured using Fisherbrand™ accuSkan™ GO UV/Vis Microplate Spectrophotometer.

Transwell Migration and Invasion Assays

Transwell cell migration and invasion assay kit (polyester inserts, BD Biosciences, Franklin Lakes, NJ, USA) were used to measure cell migration and invasion ability. Cells of KYSE510 and EC9706 cell lines were collected and cell suspension was prepared with a cell density of 4×10^4 cells/m. Cell suspension (100 µl serum-free PMI-1640 containing 4×10^3 cells) was transferred to the upper chamber, and RPMI-1640 medium containing 20% FBS was added into the lower chamber. Cell culture was performed for 24 h and membranes were collected, cleaned and stained with 0.5 % crystal violet for 20 min at room temperature. After staining, cells were observed under a light microscope (Olympus Corporation, Tokyo, Japan) and counted. Upper chamber was pre-coated with Matrigel (356234, Millipore, Billerica, MA, USA) before invasion assay.

Western Blot

RIPA solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract total proteins from cells of KYSE510 and EC9706 cell lines. BCA method was used to measure protein concentration. Proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis with 20 µg protein per lane. After gel transfer, polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham, MA, USA) were incubated with corresponding primary rabbit anti-β-catenin (1:1500, ab32572, Abcam, Cambridge, MA, USA) and anti-GAPDH polyclonal antibody (1:2000, ab181602, Abcam, Cambridge, MA, USA) overnight at 4°C. The next day, membranes were further incubated with goat anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibody (1:1500, MBS435036, MyBioSource) for 2 h at room temperature. ECL method was used to detect signal, and expression of β-catenin was normalized to GAPDH endogenous control using Image J 1.48 software (NIH, Bethesda, MD, USA).

Statistical Analysis

GraphPad Prism 7 was used for statistical analysis. NEF and β-catenin expression data were compared between two groups and among multiple groups by Student's *t*-test and one-way analysis of variance followed by LSD test, respectively. Count data were analyzed using x^2-test. $p < 0.05$ indicated a statistically significant difference.

Results

Expression of NEF in Tumor Tissues and Paired Adjacent Healthy Tissues of Esophageal Squamous-Cell Carcinoma Patients

Expression of lncRNA NEF in tumor tissues and adjacent healthy tissues of all esophageal squamous-cell carcinoma patients (n=78) was detected by qRT-PCR. According to qRT-PCR data, significantly lower expression level of NEF was found in tumor tissues than in adjacent healthy tissues in 64 out of 78 patients ($p < 0.05$), accounting for 82.1% (Figure 1), indicating that downregulation of NEF is likely involved in the pathogenesis of esophageal squamous-cell carcinoma.

Serum Levels of NEF in Patients and Controls and The Diagnostic and Prognostic Values

As shown in Figure 2a, qRT-PCR results suggest that serum levels of NEF were significantly lower in esophageal squamous-cell carcinoma patients than in healthy controls ($p < 0.05$). ROC curve analysis showed that the area under the curve (AUC) of the use of serum NEF in the diagnosis of esophageal squamous-cell carcinoma patients was 0.9042 with 95% confidence interval of 0.8547 to 0.9537 ($p < 0.05$, Figure 2b). Patients were followed up for 5 years or until their deaths. Patients with esophageal squamous-cell carcinoma were divided into high (n = 39) and low (n = 39) expression groups according to the median serum level of lncRNA NEF. Survival curves were plotted with default parameters using Kaplan-Meier method and were compared using log-rank test. As shown in Figure 2c, patients with low serum level of lncRNA NEF showed significant worse survival conditions compared with patients with high serum level of lncRNA NEF ($p < 0.001$).

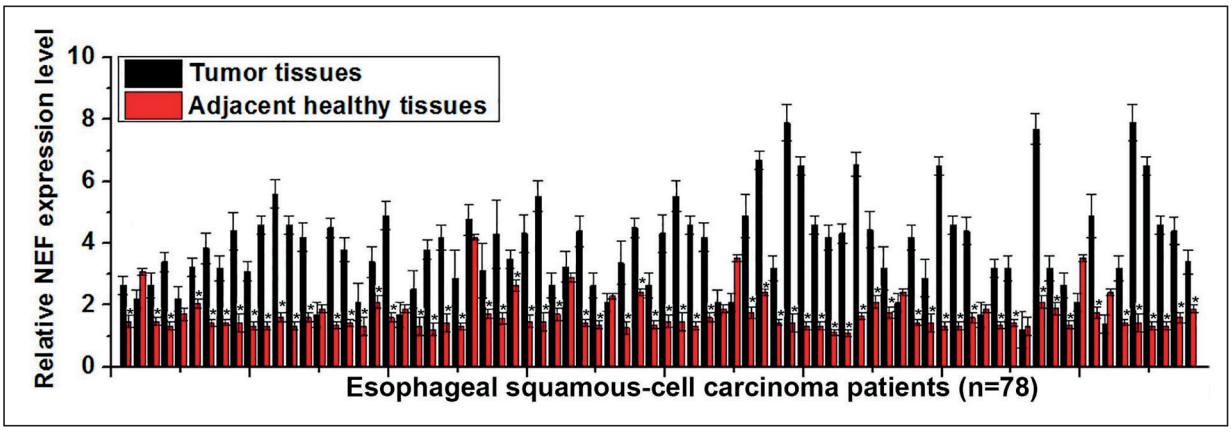

Figure 1. Expression of NEF in tumor tissues and paired adjacent healthy tissues of esophageal squamous-cell carcinoma patients. Notes: *, compared with adjacent healthy tissues, $p < 0.05$.

Correlation Between Serum Levels of NEF and Clinicopathological Data of Esophageal Squamous-Cell Carcinoma Patients

Correlation between serum levels of NEF and clinicopathological data of esophageal squamous-cell carcinoma patients was analyzed by the x^2-test. As shown in Table I, serum levels of NEF showed no significant correlations with patients' gender, age, smoking and drinking habits, but were significantly correlated with tumor size and distant tumor metastasis ($p < 0.05$).

Effects of lncRNA NEF Overexpression on β-Catenin Expression

Data in Table I suggest that NEF is likely involved in the growth and metastasis of esophageal squamous-cell carcinoma. It's known that Wnt/β-catenin pathway plays important regulatory roles in growth and metastasis of tumors[10]. In this study, NEF overexpression significantly inhibited the expression of β-catenin in cells of both KYSE510 and EC9706 cell lines (Figure 3, $p < 0.05$). However, treatment with Wnt Agonist (sc-222416, Santa Cruz Biotechnology) at different concentrations (10, 50, 100 and 200 ng/ml) showed no significant effects on NEF expression in cells of both cell lines (data not shown).

Effects of lncRNA NEF Overexpression on Cell Proliferation, Migration and Invasion of Cells of Two Esophageal Squamous-Cell Carcinoma Cell Lines

CCK-8 cell proliferation assay and transwell cell migration and invasion assays were

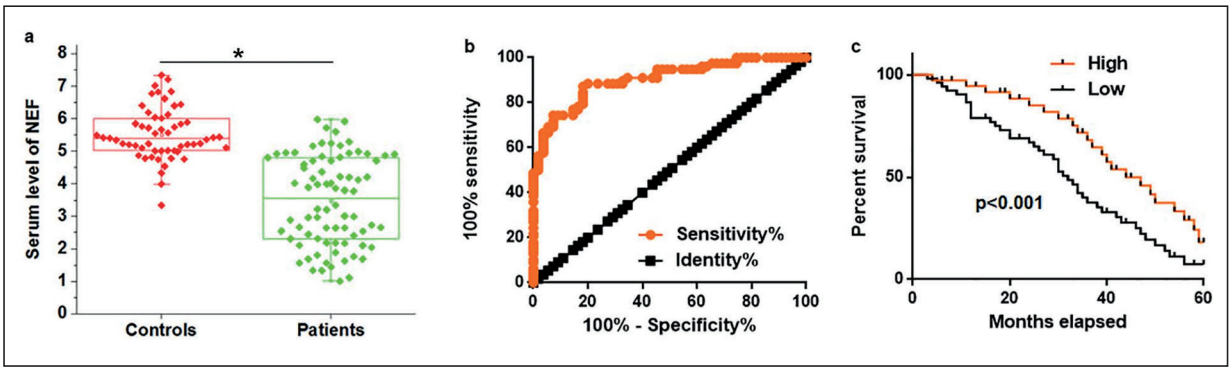

Figure 2. Serum levels of NEF in patients and controls and the diagnostic and prognostic values. This figure shows the comparison of serum levels of NEF in patients and controls ***(a)***, ROC curve of the use of serum NEF in the diagnosis of esophageal squamous-cell carcinoma ***(b)*** and comparison of survival curves of patients with high and low serum level of NEF. Notes: *, $p < 0.05$.

Table I. Correlation between serum levels of NEF and clinicopathological data of esophageal squamous-cell carcinoma patients.

Items	Groups	Cases	High-expression	Low-expression	χ^2	*p*-value
Age	> 50 (years)	42	19	23	0.83	0.32
	< 50 (years)	36	20	16		
Gender	Male	40	18	22	0.82	0.36
	Female	38	21	17		
Primary tumor diameter	> 3 cm	32	9	23	10.39	0.00
	≤ 3 cm	46	30	16		
Tumor distant metastasis	Yes	29	10	19	4.45	0.04
	No	49	29	20		
Smoking	Yes	41	20	21	0.05	0.82
	No	37	19	18		
Drinking	Yes	49	22	27	1.37	0.24
	No	29	17	12		

performed to further investigate the effects of NEF overexpression on cell proliferation, migration and invasion on esophageal squamous-cell carcinoma cells. As shown in Figure 4, NEF overexpression significantly inhibited the proliferation (Figure 4a), migration (Figure 4b) and invasion (Figure 4c) of cells of two cell lines ($p < 0.05$), while treatment with Wnt Agonist (100 ng/ml) significantly reduced the effects of NEF overexpression on proliferation, migration and invasion of esophageal squamous-cell carcinoma cells.

Discussion

In this report we observed that lncRNA NEF, a newly discovered tumor suppressor lncRNA in hepatocellular carcinoma[9], also plays a role as tumor suppressor in esophageal squamous-cell carcinoma. We also observed that the action of NEF in esophageal squamous-cell carcinoma is likely achieved through the inhibition of Wnt/β-catenin pathway. We provided a new target for the diagnosis, prognosis and treatment of esophageal squamous-cell carcinoma.

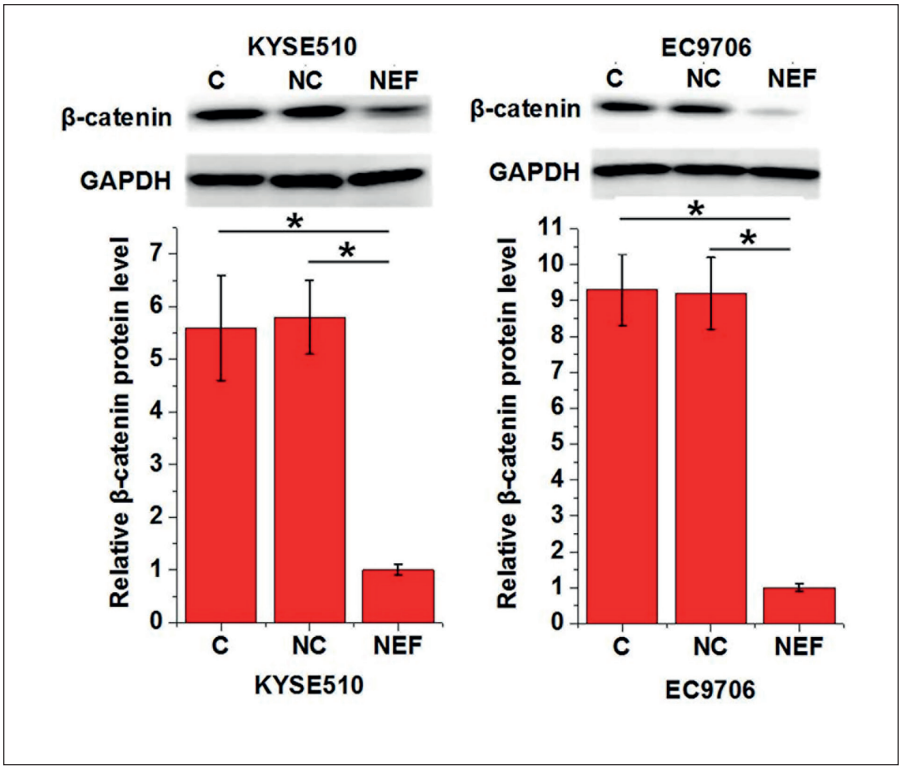

Figure 3. Effects of lncRNA NEF overexpression on β-catenin expression. Notes: *, $p < 0.05$.

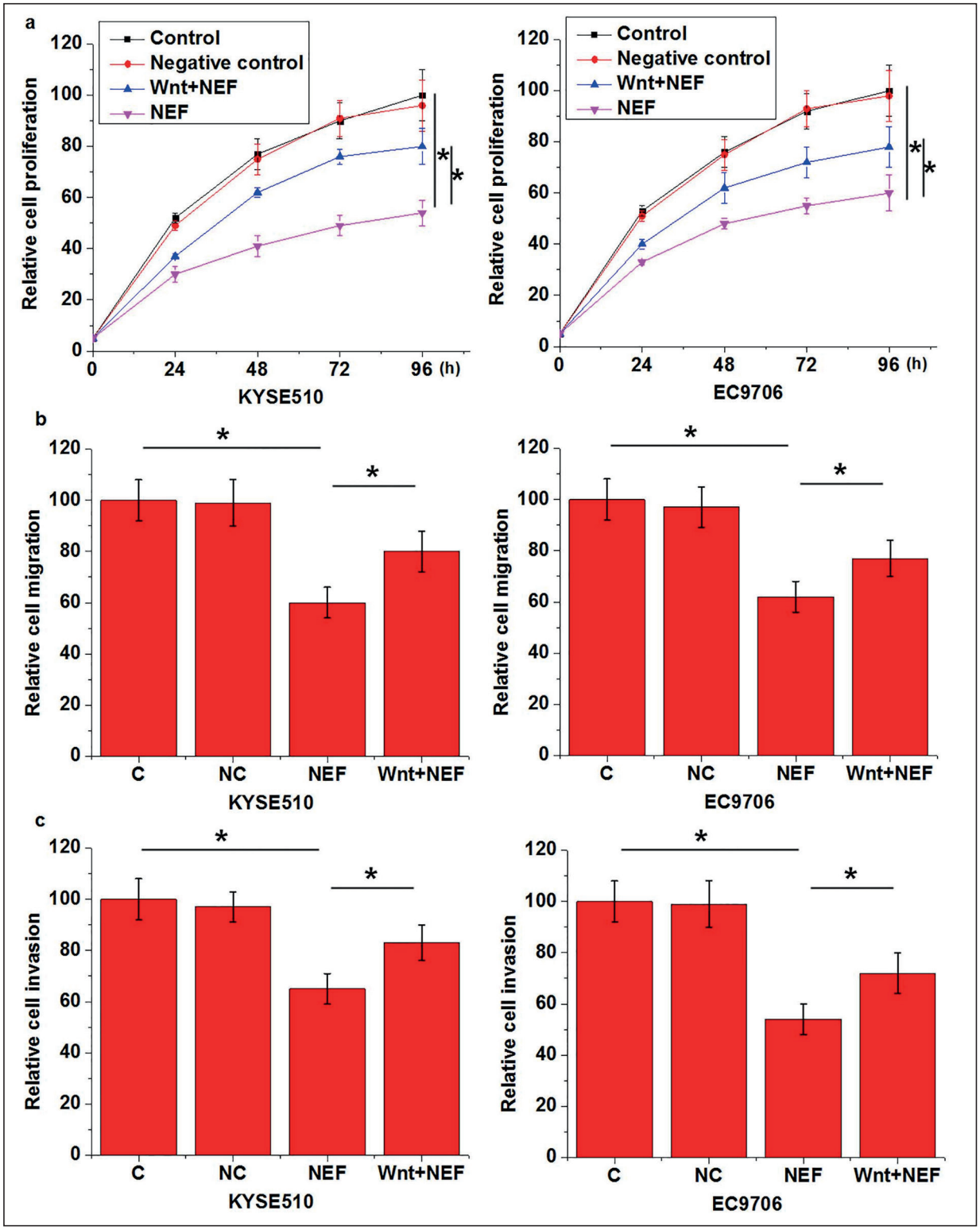

Figure 4. Effects of lncRNA NEF overexpression on cell proliferation, migration and invasion of cells of two esophageal squamous-cell carcinoma cell lines. This figure shows effects of lncRNA NEF overexpression on cell proliferation ***(a)***, migration ***(b)*** and invasion ***(c)*** of cells of two esophageal squamous-cell carcinoma cell lines. Notes: *, $p < 0.05$.

NEF performs its role as a tumor suppressor gene by inhibiting Wnt/β-catenin pathway in both esophageal squamous-cell carcinoma and hepatocellular carcinoma[10], indicating that different malignancies may share similar pathogenic pathways. LncRNAs is a group of RNA transcripts without protein-coding ability and composed of more than 200 nucleotides[11]. It has been well established that lncRNAs play pivotal roles in both physiological processes and pathological changes12. During the development of malignancies, different lncRNAs show different expression patterns to play roles as oncogene or tumor suppressor gene13. Development of esophageal squamous-cell carcinoma is also accompanied by altered expression pattern of certain lncRNAs[8]. LncRNA HOTAIR is significantly upregulated in esophageal squamous-cell carcinoma tissues than in normal healthy tissues, and overexpression of this lncRNA promotes the progression of cancer and causes poor prognosis[14]. In contrast, decreased expression of lncRNA LOC285194 in esophageal squamous-cell carcinoma tissues is a cause of failure of chemoradiotherapy, indicating the role of lncRNA LOC285194 as a tumor suppressor lncRNA in this disease[15]. Downregulated of lncRNA NEF has been observed in hepatocellular carcinoma[9]. In our study, significantly reduced expression level of NEF in esophageal squamous-cell carcinoma tissues than in paired normal healthy tissues was observed in most of patients with esophageal squamous-cell carcinoma. Therefore, our data suggest that NEF may also play a role as tumor suppressor lncRNA in esophageal squamous-cell carcinoma. We also observed that serum levels of NEF were significantly lower in esophageal squamous-cell carcinoma patients than in healthy controls. Existing of distant tumor metastasis is common in patients with esophageal squamous-cell carcinoma, leading to poor treatment outcomes and prognosis[16]. Therefore, early diagnosis and treatment is critical for the survival of patients with this disease. Changes in blood substances reflect pathogenic changes in human diseases and blood markers have been widely used to assist disease diagnosis[17]. In this work, ROC curve analysis proved that serum NEF can be used to effectively distinguish esophageal squamous-cell carcinoma patients from healthy controls. In addition, low serum NEF was also proved to be correlated with poor survival, indicating that NEF may serve as a potential diagnostic and prognostic marker for esophageal squamous-cell carcinoma. Our data suggested that serum levels of REF were not significantly correlated with age, gender as well as smoking and drinking habits, but were significantly correlated with tumor size and distant tumor metastasis. Our *in vitro* data also proved that REF overexpression promoted proliferation, migration and invasion of esophageal squamous-cell carcinoma. Wnt/β-catenin pathway plays important regulatory roles in growth and metastasis of different types of tumors[10,18-20]. Interactions between Wnt/β-catenin pathway and NEF were also observed in hepatocellular carcinoma[9]. In our study, REF overexpression inhibited the expression of β-catenin, and Wnt activator treatment reduced the effects of REF overexpression on proliferation, migration and invasion of esophageal squamous-cell carcinoma cells. Overall, our findings suggest that lncRNA-NEF may inhibit proliferation, migration and invasion of esophageal squamous-cell carcinoma cells by inactivating with Wnt/β-catenin pathway.

Conclusions

We showed that NEF was downregulated in esophageal squamous-cell carcinoma. NEF plays a role as tumor suppressor gene in esophageal squamous-cell carcinoma by inhibiting the proliferation, migration and invasion of esophageal squamous-cell carcinoma cells through the inactivation of Wnt/β-catenin pathway.

Statement of Originality and Authorship

All the authors approve the submission of this manuscript for publication into European Review for Medical and Pharmacological Sciences and have taken due care to ensure the integrity of this work. We hereby state that each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript, in accordance with the definition of an author as stated by the International Committee of Medical Journal Editors (ICMJE). We confirm that neither the manuscript nor any part of it has been published or is under consideration for publication elsewhere (abstracts excluded). Any reference to or use of previously published material protected by copyright is explicitly acknowledged in the manuscript. Posting of submitted material on any website may be considered prior publication and should be noted in the comments to the editor at the moment of submission.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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