Blockade of EEN induces apoptosis and inhibits metastasis of non-small cell lung cancer cells

Qi Song3, Yanyu Li2, Wei Sheng4, Fang Li3, Baohua Wang1

Departments of 1Thoracic Surgery, 2Gland Surgery, The Second Hospital of Hebei Medical University, Shijiazhuang 050000, Hebei, People’s Republic of China; 3Department of Oncology, Chinese PLA General Hospital, Beijing 100853, People’s Republic of China; 4College of Life Sciences, Chinese PLA General Hospital, Beijing 100853, People’s Republic of China

Received December 22, 2015; Accepted March 20, 2016; Epub April 1, 2016; Published April 15, 2016

Abstract: It has been reported that extra eleven nineteen (EEN) gene can contribute to the proliferation and survival of human multiple myeloma cells. However, the roles of EEN in human non-small cell lung cancers (NSCLC) have not been well clarified. Therefore, study investigated the biological functions and molecular mechanisms of EEN in NSCLC cell lines, discussing whether it could be a potential therapeutic target of NSCLC. In our study, we found that EEN was up-regulated in human NSCLC cell lines. Moreover, down-regulation of EEN significantly inhibited cell proliferation and induced apoptosis of H460 and A549 cells. In addition, reduced EEN protein expression suppressed invasion and EMT of H460 and A549 cells. Eventually, the effect of EEN on the Akt/mTOR signaling pathway was explored by Western blot. From our results, knockdown of EEN decreased the phosphorylation of Akt and mTOR. Taken together, EEN should be an oncogene in NSCLC. The possible mechanism was that down-regulation of EEN induced apoptosis and inhibited metastasis of H460 and A549 cells by modulating the Akt/mTOR signaling pathway.

Keywords: Non-small cell lung cancer, EEN, proliferation, apoptosis, metastasis, Akt/mTOR pathway

Introduction

Lung cancer is still the most common and the major cause of cancer death throughout the world with the highest mortality rate over the past few decades. It has been reported that its incidence rate is still increasing, especially in the China [1-3]. On the basis of previous statistics, the incidence and mortality number of lung cancer reached 1.24 million and 1.10 million in global in 2012, respectively [4]. Moreover, the corresponding incidence and mortality number were over 0.61 million and 0.49 million in 2010 in China [5]. Moreover, about 85% of lung cancer is non-small cell lung cancers (NSCLC), and the mortality of NSCLC is very high and its 5-year survival rate is less than 15% after initial diagnosis [6]. More and more evidences that numerous factors, such as environmental factors, genetic factors and their interactions, are involved in the occurrence and development of lung cancer, such as air pollution [7], smoking, chronic pulmonary disease [8] and gene polymorphisms [9, 10]. However, the potential mechanism of NSCLC pathogenesis remains unclear.

Extra eleven nineteen (EEN) gene, a SH3 domain-containing protein, is expressed ubiquitously in various tissues. The amino acid sequence of EEN is highly conserved in mammals. To this day, EEN exerted most of its roles by regulation of the location and internalization of membrane proteins. EEN is recruited to under cell membrane with other auxiliary proteins including amphiphysin, CIN85 and dynamin together during clathrin-mediated endocytosis, facilitating to shape endocytic vesicles and endosomes. Consequently, the membrane proteins are shipped into lysosomes for degradation. This feature emphasizes the importance of EEN in many biological processes such as neuroregulation, tumorigenesis and development of cancer [11-13]. Lua BL and Low BC found that EEN mediates the endocytosis of epidermal growth factor receptor (EGFR) [14]. Wu X et al also demonstrated that phosphorylated EEN at tyrosine 315 (Y315) site lost its
EEN knockdown inhibits metastasis of NSCLC cells

function to internalize membrane-locating MMP2 enzyme, leading to degrading extracellular matrix and malignant cells migrating [15]. Other studies reported that an important cause of leukemia was the fusion of EEN and MLL gene resulted from chromosome translocation [16-18]. Recently, Huang et al reported that EEN could regulate the proliferation and survival of multiple myeloma cells by potentiating IGF-1 secretion [19]. However, the roles of EEN in growth and metastasis of NSCLC cells remain to be investigated. In this study, we found that EEN regulated growth and metastasis of NSCLC cells. PI3K/AKT/mTOR pathway was involved in regulating the effect of EEN on cell growth and metastasis. These results revealed a novel role of EEN in the pathogenesis of NSCLC cells, and suggested that EEN may be a potential diagnostic marker or therapeutic target of NSCLC.

Material and methods

Cell culture

NSCLC cell lines H460, A549, H1975, H1299, PAa, Calu-3 and one human bronchial epithelial (HBE) cell line were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in RPMI 1640 (Gibco Co., New York, USA) containing 10% fetal bovine serum (FBS, Gibco Co., New York, USA), 1% penicillin and streptomycin (Beyotime Institute of Biotechnology Jiangsu, China) at 37°C in a humidified atmosphere of 5% on 0.1% gelatin-coated culture flasks (Corning, USA). Ten pairs of human lung carcinoma and their corresponding adjacent normal tissues were collected from the second hospital of HeBei Medical University. The specimens were immediately frozen in liquid nitrogen and then stored at -80°C for analysis. Prior informed consent was obtained, and the study protocol was approved by the Ethics Committee of the second hospital of HeBei Medical University.

RNA interference

Stealth siRNA duplex oligoribonucleotides 5'-UCUGUCACCCGUUGGUGTTCG-3' (siRNA) was used to against the target sequences 5'-CACCAGAAGGCGTGACAGA-3' in human EEN gene (GenBank No. NM_001199943). The siRNAs were transfected transiently with Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instructions, and a negative stealth siRNA sequence was used as a control. Briefly, siRNA was transfected by Lipofectamine™ RNAiMAX in Opti-MEMI. Then, siRNA and Lipofectamine™ RNAiMAX were combined for 5 min at room temperature, then added to the cells in quiescent state and swirled gently to ensure uniform distribution. After incubation for 6 h at 37°C, the transfection mixture was removed and the cells were further incubated in normal growth conditions for 48 h.

Reverse transcription polymerase chain reaction

Total RNA of H460 and A549 was extracted by using Trizol reagent (Life Technologies, Carlsbad, CA). Two microgram RNA was used for gene-specific reverse transcription polymerase chain reaction (RT-PCR) using one-step RT-PCR kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. Denaturation was performed at 95°C for 45 seconds, 32 cycles of 72°C for 30 seconds and 65°C for 2 minutes. The following primers were used: E-cadherin, forward 5'-TACACTGC-CCAGAGCAGCACG-3' and reverse: 5'-TGAGCAC-CAAGCAGGATTAT-3'; N-cadherin, forward: 5'-CGAATGGATGAAAGACCCATCC-3' and reverse: 5'-GGAGCCACTGCCTTCATAGTCA-3'; Vimentin, forward: 5'-GCTGAATGACCGCTTCGCCAACT'-3' and reverse: 5'-GGAGCCACTGCCTTCATAGTCA-3'; GAPDH, forward: 5'-GCTGAATGACCGCTTCGCCAACT'-3' and reverse: 5'-GAGCCACTGCCTTCATAGTCA-3'. The levels for each gene were counted by standardizing the quantified mRNA amount to the GAPDH mRNA. Each sample was assessed in triplicate.

ELISA-BrdU assay

To investigate the effect of EEN knockdown on cell proliferation of H460 and A549 cells, ELISA-BrdU assay was selected to detect the cell proliferation by using Cell Proliferation ELISA-BrdU Kit (Roche Applied Science, Mannheim, Germany) following the manufacturer's instruction. Briefly, 5×10^3 cells were seeded in 96-well plate (Corning, USA) and allowed to grow overnight in complete RPMI 1640 medium. The medium was then removed and the cells were transfected with EEN siRNA or negative siRNA control for 24 h at 37°C. After 24 h incubation, cells were additionally treated with BrdU labeling solution for the
remaining 16 h. After that, culture medium was removed, cells were fixed and DNA was denatured. Cells were incubated with Anti-BrdU-POD solution for 90 min, and then antibody conjugates were removed by washing three times. After incubation with a TMB substrate for 15 min, absorbance at 405 and 490 nm was measured to determine immune complexes.

Cell cycle analysis

The H460 and A549 cells were transfected with EEN siRNA or negative siRNA control for 24 h. Then, cells were collected by trypsinization, washed with ice-cold PBS, and fixed in ice-cold 70% methanol by incubating them for 1 h at 4°C. After that, cells were centrifuged, resuspended in ice-cold PBS, and incubated with 50 mg/ml RNase (Sigma Chemical Co., USA) for 30 min at room temperature, and then were incubated with 50 mg/ml propidium iodide (PI; Sigma Chemical Co., USA) at room temperature for 30 min. The cell cycle was analyzed by FACScan flow cytometer following the manufacturer’s guidelines (BD Biosciences, San Jose, CA, USA).

Annexin V-FITC/PI analysis

H460 and A549 cells were transfected with EEN siRNA or negative siRNA control. 24 h after transfection, cells were harvested and washed twice in PBS and double-stained with Annexin V-FITC and PI by using Annexin V-FITC Apoptosis Detection Kit (Nanjing KeyGen Biotech Co., Nanjing, China) following the manufacturer’s protocols. Then, each sample was quantitative analyzed at 488 nm emission and 570 nm excitation by FACSCalibur flow cytometer (BD, San Jose, CA, USA), and then the fluorescence was analyzed using the CellQuest software (Becton Dickinson).

Transwell invasion assay

Transwell matrigel invasion assay using Transwell chambers (8-mm pore size; Minipore) precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ) that contained extracellular matrix proteins was used to determined cell invasion. In brief, 1×10⁶ cells in 100 μl RPMI 1640 containing 1% FBS were seeded in the upper chamber, and 600 ml RPMI 1640 containing 1% FBS was added to the lower chamber. After 24 h incubation at 37°C in a 5% CO₂ atmosphere, cells that remained in the upper chamber were removed by cotton swabs and penetrating cells were fixed in methanol, and then stained with 0.1% crystal violet. Cell invasion was quantified by counting cells on the lower surface using phase contrast microscopy.

Western blot analysis

H460 and A549 cells were washed twice in cold PBS, and then lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology Jiangsu, China). The protein concentration of cell lysates was quantified by BCA Kit (Beyotime Institute of Biotechnology Jiangsu, China), and 50 μg of each of proteins were separated by SDS-PAGE on 10% gels, and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blocked in 5% non-fat milk diluted with Tri Buffered Saline Tween-20 (TBST) at room temperature for 1 h and incubated overnight at 4°C with primary antibody respectively: anti-EEN (1:500; Santa Cruz Biotechnology, CA, USA), anti-p21, anti-cyclin D1, anti-CDK4, anti-Bax and anti-PCNA (1:1000; Cell Signaling Technology Inc, MA, USA). The membranes were then incubated with a goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase secondary antibody for 2 h. The proteins were visualized using ECL-plus reagents (Amersham Biosciences Corp., USA). The density of the bands was measured using the Image J software (USA), and values were normalized to the densitometric values of α-tubulin in each sample.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., USA). Data from each group were expressed as mean ± standard error of the mean (S.E.M.) and statistically analyzed by Student’s t test. Differences were considered statistically significant at a p value of <0.05.

Results

The expression of EEN is increased in NSCLC tissues and cell lines

Since the level of EEN in NSCLC remains unknown, we were aim to use qRT-PCR to
detect the level of EEN in NSCLC tissues and cell lines. We found that the levels of EEN in the NSCLC tissues were increased and evidently higher in comparison to the adjacent tissues (Figure 1A). To determine the level of EEN in NSCLC cells, the level of EEN was detected in a human bronchial epithelial cell (HBE) and six NSCLC cell lines including H460, A549, H1975, H1299, PAa and Calu-3. We found that the level of EEN was significantly increased in these seven NSCLC cell lines compared to that in human bronchial epithelial cell HBE, as shown in Figure 1B. Among these NSCLC cell lines, H460 and A549 cells were used to study further.

**Effects of EEN knockdown on cell proliferation, cell cycle and apoptosis in both H460 and A549 cells**

Due to up-regulation of EEN in human NSCLC cells, we supposed that EEN might function as an oncogene. After EEN knockdown, we found that expression of EEN protein was significantly decreased in EEN siRNA group compared to negative siRNA control group (Figure 2A). Next, to explore the role of EEN in proliferation of NSCLC cells, the results of BrdU-ELISA assay showed that down-regulation of EEN had available anti-proliferative effect in both H460 and A549 cells (Figure 2B).

Since EEN knockdown significantly inhibited proliferation of NSCLC cells, we speculated that down-regulation of EEN could arrest the cell cycle of NSCLC cells. Detecting by flow cytometry, our results demonstrated that down-regulation of EEN dramatically increased the percentage of cells in the G1-phase in both H460 and A549 cells (Figure 2C). Therefore, EEN knockdown might inhibit the proliferation of NSCLC cells by hindering the transition of cell cycle from G1 phase to S phase.

In order to further study whether EEN knockdown exerted its anti-proliferative effect through induction of cell apoptosis, the total apoptosis rates of H460 and A549 cells were also detected by flow cytometry analysis. We confirmed that the apoptotic rate of H460 and A549 cells was higher in EEN siRNA group than that in negative siRNA control group (Figure 2D).

**The effects of EEN knockdown on the expressions of cell proliferation, cell cycle and apoptosis-related proteins in NSCLC cells**

The cell proliferation and cell cycle were inhibited, and the apoptosis was induced in H460 and A549 cells after EEN knockdown. Thus, these related-proteins including PCNA (a proliferation marker), p21 protein (a cyclin-dependent kinase inhibitor), CDK4 (a cyclin-dependent kinase), cyclin D1 (a cell cycle protein), and Bax (a pro-apoptotic protein) were determined by Western blot analysis. We found that the expressions of PCNA, CDK4 and cyclin D1 dis-
played obvious down-regulation in EEN siRNA group than those in the negative siRNA control group (Figure 3). However, expressions of p21 and Bax proteins were significantly up-regulat-
EEN knockdown inhibits metastasis of NSCLC cells

Figure 3. The effects of EEN on the expressions of cell proliferation, cell cycle and apoptosis-related proteins in NSCLC cells. H460 and A549 cells cells were transfected with EEN siRNA or negative siRNA control for 24 h. The protein expressions of PCNA, p21, cyclin D1, CDK4, and Bax in H460 and A549 cells were determined by Western blot, respectively. α-tubulin was detected as a loading control. All data are presented as mean ± SEM, n=6. *P<0.05, **P<0.01, ###P<0.001 vs. NC.

ed by down-regulation of EEN (Figure 3). These results indicated that EEN knockdown might be associated with the down-regulation of PCNA, CDK4 and cyclin D1, and the up-regulation of Bax, and p21 in H460 and A549 cells.

Down-regulation of EEN inhibited the invasion of NSCLC cells

To know the role of EEN in invasion of NSCLC cells, we evaluated the invasive capacities of H460 and A549 cells after EEN knockdown by Transwell invasion assays. The data from Transwell assays showed that the invasion capability of H460 and A549 cells was significantly inhibited in EEN siRNA group compared to negative siRNA control group (Figure 4). These data confirmed that EEN knockdown might play a critical role in inhibition of invasion in H460 and A549 cells.

Effects of EEN knockdown on EMT-related proteins of NSCLC cells

To know whether EMT contributed to inhibition of NSCLC cell invasion by down-regulation of EEN, we explored the effects of EEN knockdown on the expressions of EMT markers in H460 and A549 cells using qRT-PCR. Down-regulation of EEN in H460 and A549 cells lead to up-regulation of the epithelial marker E-cadherin, and down-regulation of the mesen-
EEN knockdown inhibits metastasis of NSCLC cells

chymal markers N-cadherin and Vimentin at mRNA levels (Figure 5). Taken together, our data showed that EEN knockdown could suppress the invasive ability of NSCLC cells partly by regulation of EMT.

**EEN knockdown inhibited growth and metastasis of NSCLC cells through PI3K/Akt/mTOR pathway**

To further investigate the molecular mechanism of EEN on the growth and metastasis in NSCLC cells, we determined the effect of EEN knockdown on the PI3K/Akt/mTOR pathway. The association of PI3K/AKT/mTOR pathway with human cancer has been revealed by numerous studies since 1990s. After down-regulation of EEN, the phosphorylation of Akt and mTOR in both H460 and A549 cells were determined by Western blotting. In both NSCLC cells, knockdown of EEN decreased Akt and mTOR phosphorylation in H460 and A549 cells (Figure 6). However, there are no effects on total Akt and mTOR expressions (Figure 6). Collectively, these data indicated that EEN knockdown inhibited proliferation of both H460 and A549 cells by regulating PI3K/AKT/mTOR pathway.

**Discussion**

The role and its mechanism of EEN in NSCLC remain unknown. In our study, the results showed that EEN was frequently up-regulated
EEN knockdown inhibits metastasis of NSCLC cells

in NSCLC tissues and cell lines. According to these data, we speculated that EEN might be a potential oncogene in NSCLC. As expected, down-regulation of EEN suppressed growth and metastasis of H460 and A549 cells. Our findings indicated that EEN played critical roles in regulation of growth and metastasis in NSCLC cells and may be potential diagnostic and predictive biomarkers.

Next, we investigated the mechanism of EEN in inhibiting proliferation, invasion and inducing apoptosis in NSCLC cells. In the present study, results from Brdu-ELISA assay showed that knockdown of EEN could dramatically inhibited the proliferation of H460 and A549 cells. Cell cycle analyses also showed that the percentage of cells in the G1-phase was increased and the percentage of cells in the S-phase was decreased in cells transfected with EEN siRNA compared to cells transfected with negative siRNA control. Moreover, flow cytometry analysis confirmed that EEN knockdown could significantly promoted apoptosis of H460 and A549 cells. It has been well known that cell proliferation, cell cycle progression and apoptosis are regulated by multiple proteins. To confirm the possible mechanisms of EEN on regulation of cell proliferation, cell cycle and apoptosis, we investigated the effects of EEN knockdown on cell proliferation-, cell cycle- and apoptosis-related proteins. We detected the expressions of PCNA, p21, cyclin D1, CDK4 and Bax. From our data, we found that down-regulation of EEN decreased the protein levels of PCNA, cyclin D1, CDK4 and increased p21 expression, respectively. Cyclin D1 interacts with CDK4 to form the cyclin D-CDK4 complex, and then phosphorylates Rb, which plays a critical role in carcinogenesis. The cyclin D1/CDK4/p-Rb

Figure 6. Knockdown of EEN inhibited cell proliferation, invasion and EMT by inactivating Akt/mTOR signaling pathway. H460 and A549 cells were transfected with EEN siRNA or negative siRNA control. Western blot analysis was performed for p-Akt, t-Akt, p-mTOR and t-mTOR, respectively. All data are presented as mean ± SEM, n=6.

**P<0.01, ***P<0.001 vs. NC.
EEN knockdown inhibits metastasis of NSCLC cells

pathway has been proved to be changed in most of human cancers before [20, 21]. It is a pivotal regulator of the G1 to S phase transition of the cell cycle. However, p21, a member of the Cip/Kip family, blocked cell cycle progression via inhibiting the activity of the cyclin-CDK complexes [22]. Bax, a pro-apoptotic protein, stimulates mitochondrial damage and promotes apoptosis [23]. In this study, our data showed that EEN knockdown increased expression of Bax protein, which suggested that EEN regulated cell apoptosis by modulation of Bax. Altogether, our findings demonstrated that EEN affected cell proliferation, cell cycle and apoptosis by regulating PCNA, p21, cyclin D1, CDK4 and Bax.

In addition, Transwell assay showed that EEN knockdown dramatically inhibited the invasion of H460 and A549 cells compared with negative siRNA control group. Furthermore, another process of metastasis is EMT, so we determined the levels of EMT-related gene in H460 and A549 cells transfected with EEN siRNA. Our results showed that down-regulation of EEN could markedly suppress EMT of NSCLC cells by dramatically up-regulating the epithelial marker E-cadherin and down-regulating the mesenchymal marker N-cadherin and Vimentin. Above data showed that decreased EEN expression might suppress invasion and EMT processes to restrain cell metastasis.

PI3K/Akt/mTOR pathway is one of the most critical intracellular signaling pathways. By affecting the activation status of multiple effector molecules in its downstream, this pathway can regulate series of biological processes including the proliferation, apoptosis, metastasis, and metabolism of cells [24, 25]. Recently, the abnormal activation of PI3K/Akt/mTOR pathway has been found in numerous human malignancies. At the same time, the activation of this pathway may also perform a critical role in the excessive proliferation and blocked apoptosis of cancer cells [26, 27]. The activation of Akt can inhibit cell apoptosis and promote the survival of tumor cells. In multiple malignant cancers such as breast cancer and NSCLC, the PI3K/Akt pathway is activated to counteract the cell apoptosis caused by radiotherapy and/or chemotherapy [28, 29]. Besides, mTOR can transfer the mitotic signals to p70S6k, upregulating the translation of the major cell-cycle-related proteins such as cyclin and cyclin-dependent kinase (e.g. CDK4), and then inhibiting the expressions of CDK4 inhibitors (e.g. p21CIP1), leading to promoting G1 progression, accelerating cell cycle, stimulating cell proliferation and differentiation, and promoting the occurrence and development of cancers [30, 31]. As shown in previous studies, EEN was closely related to the proliferation, cell cycle and apoptosis of multiple myeloma cells, and the transfection of recombinant vector pcDNA3.1-EEN into cells could evidently activate the Akt/mTOR pathway, whereas EEN siRNA significantly inactivated the Akt/mTOR pathway [19]. In our present study, in NSCLC cells with knockdown of EEN expression, the protein expressions of Akt, p-Akt (Ser473), mTOR and p-mTOR (Ser2448), were determined by Western blotting. We found that the knockdown of EEN inhibited the phosphorylation of Akt and mTOR. Therefore, EEN might exert its biological effect on NSCLC cells via the PI3K/Akt/mTOR pathway.

In conclusion, our data have showed that level of EEN mRNA was significantly up-regulated in NSCLC tissues and cells. Down-regulation of EEN inhibited proliferation, invasion and induced apoptosis of NSCLC cells by regulating PI3K/AKT/mTOR pathway. This novel finding might provide new insights into the molecular mechanisms underlying progression and metastasis of NSCLC, and down-regulation of EEN might be a possible therapeutic strategy for NSCLC treatment in the future.

Disclosure of conflict of interest
None.

Address correspondence to: Dr. Baohuang Wang, Department of Thoracic Surgery, The Second Hospital of Hebei Medical University, No. 238 West Heping Road, Shijiazhuang 050000, Hebei, People’s Republic of China. Tel: +86 311 85878286; Fax: +86 311 85878286; E-mail: wangbaohuahebei@163.com; Dr. Fang Li, Department of Oncology, Chinese PLA General Hospital, No. 28 Fuxing Road, Haidian District, Beijing 100853, People’s Republic of China. E-mail: doctorlifang301@163.com

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EEN knockdown inhibits metastasis of NSCLC cells


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EEN knockdown inhibits metastasis of NSCLC cells


