Lung Cancer 124 (2018) 31–39

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Elongation factor-2 kinase (eEF-2K) expression is associated with poor patient survival and promotes proliferation, invasion and tumor growth of lung cancer

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ARTICLE INFO

Keywords:
Eukaryotic elongation factor-2
eEF-2 kinase
Lung cancer
Liposomes
Rottlerin
siRNA
Signaling
Survival
Tumorigenesis

ABSTRACT

Objectives: Lung cancer is the leading cause of cancer related deaths in worldwide. Despite recent advances in treatment options, patient survival has not improved substantially due to lack of commonly expressed molecular targets and effective targeted therapeutics. Thus, better understanding of the biology of lung cancer and identification of novel therapeutic targets are urgently needed for development of highly effective molecularly targeted therapies.

Materials and methods: Viability, proliferation and metastatic ability of lung cancer cells were evaluated using methylthiazoltetrazolium (MTT), colony formation and matrigel invasion assays, respectively. Western blotting, RT-PCR, and gene knockdown by siRNA transfections were carried out to investigate the effects of eEF-2K on lung cancer cells. Athymic Nu/Nu mice were treated with liposomal eEF-2KeEF-2K or control siRNA and tumor growth was evaluated in tumor xenograft models of lung cancer.

Results and discussion: Here, we report that Eukaryotic Elongation Factor-2 kinase (eEF-2K), a member of an atypical alpha kinases family, is significantly upregulated in lung cancer cell lines and its expression is associated with shorter overall patient survival in lung cancer. Inhibition eEF-2K expression by siRNA or a chemical inhibitorsignificantly suppressed lung cancer cell proliferation, colony formation, survival, migration/invasion and tumorigenesis by inhibiting cyclin D1, Src and Mitogen-Activated Protein Kinases/Extracellular Signal-Regulated Kinase (MAPK/ERK) signaling. In vivo targeting of eEF-2K by systemically injected nanoliposomal eEF-2K siRNA resulted in a significant inhibition of lung cancer tumor xenografts in nude mice. Our results suggest, for the first time, that expression of eEF-2K is associated with poor patient prognosis and involved in regulation of critical pathways, including Src and MAPK/ERK and cyclin D1, promoting tumor growth and progression, and thus may be a novel potential therapeutic target in lung cancer.

1. Introduction

Lung cancer is the leading cause of cancer-related death both in USA and accounts for 1.8 million new cases (13.0% of the all cancers) cases each year in worldwide [1,2]. Epithelial lung cancer is classified into two main groups: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is the most common subtype of lung cancer and accounts for more than 80% of the total lung cancer occurrence [3]. NSCLC comprises squamous-cell carcinoma (SCC), adenocarcinoma (ADC), and large-cell lung cancer (LCC) [4]. Current therapy includes surgical removal if not improbable, definitive/palliative radiotherapy or cytotoxic chemotherapy as well as targeted therapies including angiogenesis inhibitor (bevacizumab) [5], epidermal growth factor receptor (EGFR) inhibitors such as afatinib [6], erlotinib [7], gefitinib [8], osimertinib [9]), ALK kinase inhibitors (alectinib [10], ceritinib [11], crizotinib [12]), ROS1 rearrangement (ceritinib [13], crizotinib

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https://doi.org/10.1016/j.lungcan.2018.07.027
Received 23 May 2018; Received in revised form 19 July 2018; Accepted 19 July 2018

[14]), BRAF V600E mutations (dabrafenib/trametinib) [15], and checkpoint inhibitors (pembrolizumab [16], atezolizumab [17], nivolumab [18]). Immunotherapy based checkpoint inhibitors such as anti PD-L/PD-L1 and anti CTLA4 antibodies seemed promising in clinical trials, inducing objective response in 20% of lung cancer patients with 6 months of median stable disease [19]. Despite all these recent advances in therapeutic approaches, patient survival rate is extremely poor (8–12 months median survival and 17.4% 5 year survival) and has not improved substantially [20]. Therefore, there is an urgent need to identify common molecular targets and develop novel therapeutic targets to improve poor survival in patients with lung cancer patients.

eEF-2K, an atypical member of alpha kinase family with calcium/calmodulin-dependent Ser/Thr-kinase activity, is overexpressed in cancer cells, including breast, pancreatic and glioblastome multiforme [21–33]. eEF-2K is activated under stress conditions such as hypoxia, growth factor and nutrient or energy depletion and autophagy, suggesting that eEF-2K acts as a cell survival pathway and functions as a cytoprotective factor in cancer cells by regulating peptide elongation phase of protein synthesis through phosphorylation of eEF-2 [21,25,22–33]. The activation of eEF-2K is often found to be increased in rapidly proliferating malignant cells and correlated with higher proliferation rate [27,33–35]. Currently the role of eEF-2K in lung cancer is unknown.

In the current study, we found that eEF-2K expression is associated with poor patient survival by the analysis of lung cancer patient databases. Thus we hypothesized that eEF-2K promotes tumor growth and progression by inducing proliferation, invasion/ metastasis ability of lung cancer cells and we evaluated its role and molecular mechanisms involved in proliferation, survival, invasion of lung cancers. We found that eEF2 K expression promotes lung cancer tumorigenesis by inducing cyclin D1, Src and Mitogen-Activated Protein Kinases/Extracellular Signal-Regulated Kinase (MAPK/ERK) signaling in lung cancer and may serve as potential molecular target.

2. Material and methods

2.1. Cell lines, culture conditions and reagents

All lung cancer cell lines (A549, H1299, H322, H292 and H226) were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and maintained in RPMI-1640 media (Gibco-Invitrogen, Carlsbad, CA, USA), supplemented with 10% of fetal bovine serum and 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). Human bronchial epithelium cells (HBEb) were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and maintained in Bronchial Epithelial Cell Growth Medium (BEGM) (Lonza Walkersville Inc., Walkersville, MD, USA), which was supplemented with the contents of the BEGM™ SingleQuots™ Kit (Catalog No. CC-4175) containing bovine pituitary extract, 2.0 ml; hydrocortisone, 0.5 ml; human epidermal growth factor, 0.5 ml; epinephrine, 0.5 ml; transferrin, 0.5 ml; insulin, 0.5 ml; retinoic acid, 0.5 ml; triiodothyronine, 0.5 ml; gentamicin /amphotericin-B, 0.5 ml). Cells between passages 4 and 15 were used for experiments, seeded into Falcon tissue culture dishes and cultured at 37 °C in a humidified incubator with 5% CO_2. Rottlerin was purchased from (Sigma-Aldrich, St. Louis, MO), dissolved in dimethyl sulfoxide (DMSO) and directly added to the cell cultures at indicated concentrations (μM). Control cells were treated with DMSO alone.

2.2. In vitro cell viability and proliferation assays

The proliferation of human lung cancer cells was assayed with MTS [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] reduction. Viable cells counted using trypan blue dye in a hemocytometer were seeded in 96-well plates as 1.25×10^3cells/well. After incubation period for overnight, cells were treated with rottlerin or siRNAs (control or eEF-2K siRNA) for 4 days or 5 days, respectively. Formazan product generated from MTS via dehydrogenases within viable cells was quantified at 490 nm. All experiments were performed in triplicate and the results were expressed as mean of absorption ± standard deviation.

2.3. Colony formation assay

A549 cells seeded (1×10^3 cells per well) into 6-well plates were treated with of rottlerin at indicated doses or 50 nM siRNAs (Control, eEF-2K#1, eEF-2K#2), once a week, for 14 days under regular culture conditions. The colonies formed in the wells were stained with 10% crystal violet.

2.4. Transfections with siRNA

siRNAs targeting eEF-2K or control siRNA were purchased from Sigma-Aldrich (St. Louis, MO). eEF-2K siRNA#1, 5′-GCCAACCAGUAC UACCAAA-3′, eEF2 K siRNA#2, 5′-AAGCUCGAACCAGAAUGUC-3′ [21,36], control non-silencing siRNA (5′-AAUUCUCCGAACGUGUCA CGU-3′) [21,36,37] were employed to transfect the cells with each siRNAs, at a final concentration of 50 nM for 72 h, using HiPerFect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose response studies.

2.5. Matrigel invasion assay

The cells were treated with eEF-2K inhibitor rottlerin [21,36] or DMSO as a control for 6 h, or transfected with 50 nM of eEF-2K or control siRNAs for 24 h. After treatment period, fixed number of treated cells (5×10^4), were seeded onto matrigel-coated transwell filters (8-mm pore size) in matrigel invasion chambers (BD Biosciences, San Jose, CA) with chemoatractant (media with FBS) in the lower well. After incubation period (24 h for rottlerin treatments and 48 h for siRNA transfections), the filter is removed, fixed, and stained. The number of cells that invaded the lower side of the filter was quantitated by counting cells in a minimum of five randomly selected microscopic fields [38]. The experiment was performed in triplicate and the results were reported as a mean number ± standard deviation.

2.6. Western blot analysis

Cells were seeded in T-25 tissue culture flasks (0.5×10^6 cells/flask) and collected after treatments, centrifuged, washed twice in ice cold phosphate-buffered saline (PBS) and whole-cell lysates were obtained by suspending the cells in a lysis buffer at 4 °C. Lysates were centrifuged at 13,000 *g* for 10 min at 4 °C, and the supernatant fractions were collected. Protein concentration for each sample was determined by Bradford assay (Bio-Rad, Hercules, CA), and Western blotting was performed. The membranes were blocked with blocking buffer (0.1% Triton X-100) and 5% dry milk and probed with the following primary antibodies: (diluted in Tris-buffered saline-Tween 20 (TBS-T) containing 2.5% dry milk, and incubated overnight at 4 °C with primary antibodies of eEF-2K, p-eEF-2 (Thr-56), p-Src (Tyr-416), Src, p-ERK, Cyclin D1, β-Tubulin (Cell Signaling Technology, Danvers, MA). The membranes were washed with TBS-T and incubated with horseradish peroxidase–conjugated anti-rabbit or anti-mouse secondary antibody (Cell Signaling Technology, Danvers, MA). Chemiluminescent detection was performed with Chemi-glow detection reagents (Alpha Innotech, San Leandro, CA). The blots were visualized with a FluorChem 8900 imager (Protein Simple, San Jose, CA) and quantified by a densitometer using the Alpha Imager application program (Alpha Innotech, San Leandro, CA). Mouse anti-b-actin (primary) and anti-mouse (secondary) antibodies (Sigma Chemical, St. Louis, MO) were used to monitor B-actin expression as a loading control.

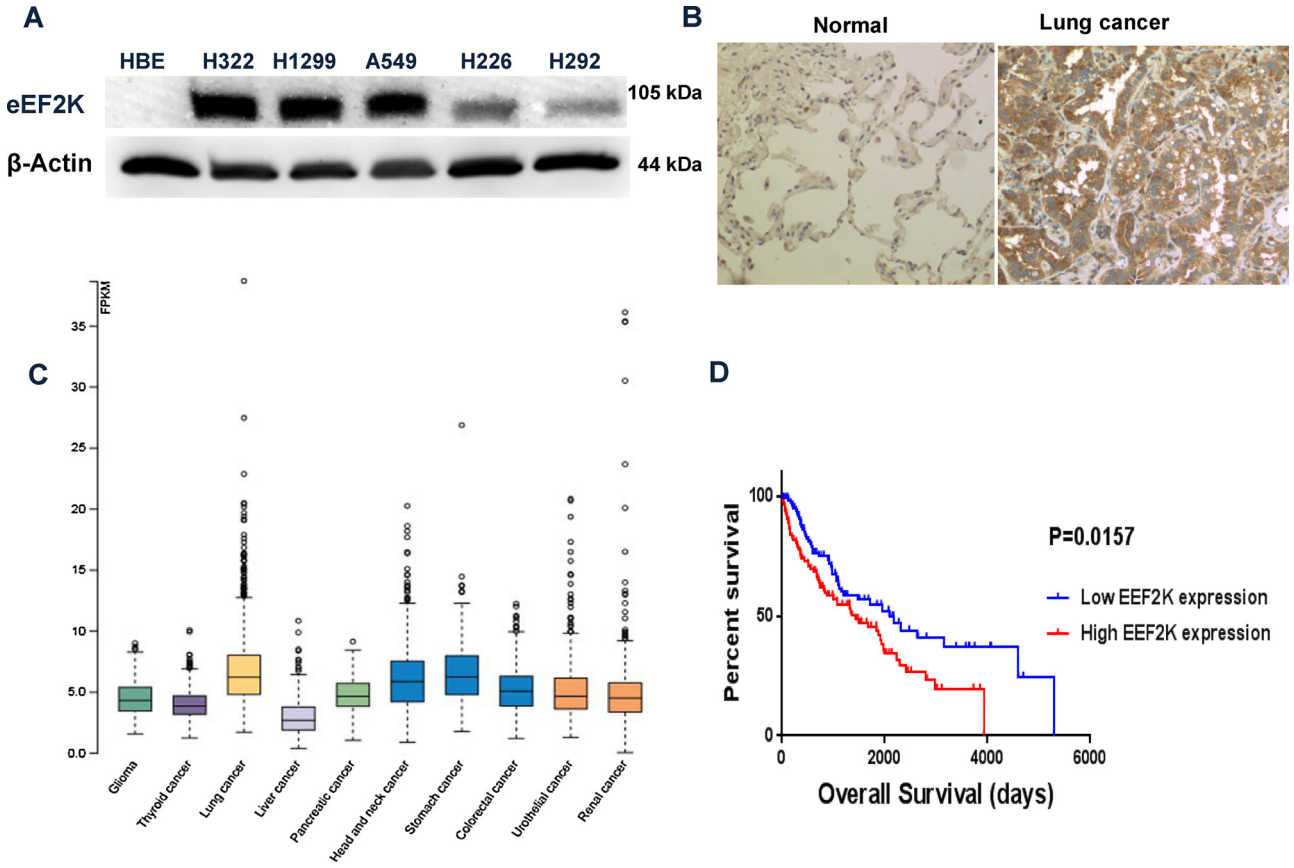

Fig. 1. A. Expression of eEF-2K is upregulated in lung cancer cell lines. eEF-2K expression is markedly higher in lung cancer cells compared to normal human bronchial epithelial cells (HBE) detected by Western blot. B. eEF-2K is overexpressed in lung cancer patient tumors compared to normal lung tissues detected by immunohistochemistry (IHC). C. Relative eEF-2K mRNA expression levels in various cancer patients based on TCGA-pan-cancer database. D. Kaplan-Meier survival analysis shows that high eEF-2K mRNA expression levels predict poor prognosis in patients with lung cancer. eEF-2K mRNA levels are associated with shorter survival time in lung cancer patients based on GEO lung cancer tissue database.

2.7. RNA isolation and reverse transcriptase–polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was isolated using TRIzol® Reagent (Invitrogen/Life Technologies, Carlsbad, CA) lung cancer cells. 1 μg of total RNA was used to synthase cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The cDNA for eEF-2K and Integrin-β1 were amplified using Platinum Taq DNA Polymerase kit (Invitrogen/Life Technologies), with specific primers. Briefly, 2 μL cDNA was used for PCR in 1x PCR buffer containing 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates (dNTPs), 1 unit of Platinum Taq polymerase, and 0.2 μM of each eEF-2K and Integrin-β1 primers (Integrated DNA Technologies, IDT), or GAPDH specific primers (Thermo Scientific). The sequences of the sense and anti-sense eEF2 K primers are 5′-GCAATC AGCCAAGACCATCT-3′ and 5′-GGAGAGAGTCGAAGGTCACG-3′, respectively. The cDNA samples were incubated at 94 °C (2 min.) to denature the template and activate the enzyme. This step was followed by 35 cycles of PCR amplification (in each cycle, the samples were incubated at 94 °C for 30 s., 60 °C for 30 s. and 72 °C for 60 s.) with an additional cycle at 72 °C for 5 min. The amplified reaction products were analyzed on a 1.2% agarose gel containing ethidium bromide. The cDNA synthesis was verified by detection of the GAPDH transcript, which was used as an internal control.

2.8. Preparation of siRNA liposomal nanoparticles

For in vivo eEF-2K and control siRNA treatments, siRNA was incorporated into nanoliposomes made of Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and pegylated distreroly-phosphotidyl ethanolamine (DSPE-PEG-2000) (Avanti Lipids) at the ratio of (10:1) and mixed with siRNA at a ratio of 10:1 (lipid to control siRNA or eEF-2K siRNA) (w/w) in the presence of excess tertiary butanol and lyophilized overnight. Prior to injection to mice, liposomes were reconstituted in PSB and injected (i.v from tail vein of mice) in a volume of 100 μL.

2.9. In vivo tumor xenograft models of lung cancer

Athymic Nu/Nu female mice (4–5 week old) were obtained from the Department of Experimental Radiation Oncology at M. D. Anderson Cancer Center, Houston, TX. All studies were conducted according to a protocol approved by the M. D. Anderson Institutional Animal Care and Use Committee. H1299 and A549 cells (3×10^6 in 20% matrigel) were injected into right flank of each mouse. Two weeks after injection, when tumors reached about 3–5 mm in size, liposomal siRNA treatments (I.V from tail vein) were initiated. Each mouse received 0.3 mg/kg siRNA (control or eEF-2K siRNA) or equivalent of 8 μg/mouse incorporated in liposomes once a week for four weeks and tumor volumes were measured weakly by a caliper. After completion of treatments, mice were euthanized with CO_2, tumor tissues were removed, lysed and analyzed.

2.10. Immunohistochemistry

Formalin-fixed, paraffin-embedded tumor samples from the tumors resected from the mice were sectioned (5 μm) and stained with hematoxylin and eosin. Immunostaining for Ki-67 was performed to evaluate cell proliferation, according to the manufacturer's protocol. The slides

were analyzed by microscopy (Nikon Eclipse TE-200-U; Nikon Instruments, Inc., Melville, NY).

2.11. Statistical analysis

All experiments were conducted at least in triplicate, and the data was expressed as the means with standard deviations of these independent experiments. Statistical significance was determined using the Student *t*-test P-values less than 0.05 were considered statistically significant.

3. Results

3.1. eEF-2K is overexpressed in lung cancer cells and associated with shorter patient survival

Lung carcinoma is characterized by high proliferation rates, extensive local tumor invasion, early systemic dissemination and poor patient survival. eEF-2K expression is highly expressed in cells with high proliferating rate and is associated with viability and cellular survival mechanisms [27,33,36]. To investigate eEF-2K expression in lung cancer cell lines, we evaluated eEF-2K expression in five different types of non-small cell lung cancer cell lines (H1299, A549, H226, H322 and H292) compared with normal bronchial epithelium cell line (HBE) by Western blot analysis. Among lung cancer cells, the most prominent eEF-2K expression was detected by A549, H1299 and H322 cells (Fig. 1A). eEF-2K protein and gene expressions were found to be upregulated in another set of lung cancer cell lines (Supplementary Fig. 1A,B respectively) and in a panel of cells according to Cancer Cell Line Encyclopedia (CCLE) analysis (Supplementary Fig. 2A,B). eEF-2K expression was also confirmed in lung cancer patient samples by immunohistochemistry (IHC) (Fig. 1B). More importantly, eEF-2K gene expression highly upregulated in lung cancer patients compared to other cancers based on the mRNA analysis of TCGA-pan-cancer database (https://www.proteinatlas.org/ENSG00000103319-EEF2K/pathology) (Fig. 1C) [39]. Our analysis of human lung cancer patient database (GEO dataset ID: GSE36471- https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36471 [39]) revealed that eEF-2K gene expression is associated with shorter patient overall survival and poor clinical outcome (Fig. 1D).

3.2. Inhibition of eEF-2K expression suppresses cell proliferation and colony formation in lung cancer cells

To investigate the role of eEF-2K in lung cancer cell proliferation and colony formation, we inhibited its expression genetically by RNA-interference (siRNA) using two different eEF-2K specific siRNA sequences [21,22] and a chemical inhibitor (Rottlerin) [26,31]. We showed that eEF-2K siRNAs (50 nM) and rottlerin treatments (5 and 10 μM) inhibited expressions of eEF-2K protein (Fig. 2A, B) and mRNA (Fig. 2C, D) in A549 cells using western blot and RT-PCR analysis, respectively. Later studies demonstrated that eEF-2K siRNAs significantly inhibited cell proliferation and colony formation of lung cancer cells that expresse eEF-2K, including A549 and H1299 cells, by about 40% to 70% (72 h), respectively, compared to control siRNA (Fig. 3A–E). We did not detect significant inhibition of cell proliferation in cells that express low eEF-2K such as H292 (Supplementary Fig. 3). As expected rottlerin (5 and 10 μM) treatment also inhibited cell proliferation and colony formation of lung cancer cells (Fig. 3F,G).

3.3. Knockdown of eEF-2K impairs invasion and migration of lung cancer cells

Metastasis is the major cause of lung cancer related deaths. Considering the clinical significance of eEF-2K in poor patient survival we investigated the role of eEF-2K on cell invasion and migration of lung cancer cells by inhibiting its expression by siRNA or rottlerin and evaluated the effects in lung cancer cells by performing in vitro invasion assay using matrigel-coated Boyden chambers [26,31]. The assay measures the number of cancer cells migrating and invading through a basement membrane matrix (matrigel) towards media containing chemo-attractants [38]. We found that knockdown of eEF-2K by two different siRNA sequences significantly decreased migration and invasion of A549 cells by about 51.2% and 52.4%, respectively (Fig. 4A, B) and H322 lung cancer cell line with high eEF2 K expression (Supplementary Fig. 4A–C). Rottlerin (5 μM and 10 μM) treatment also markedly reduced invasion of A549 cells with a dose dependent manner by about 61% and 70%, respectively (Fig. 4C, D). These results overall suggest that inhibition of eEF-2K impairs the migration/invasion capacity of lung carcinoma cells.

3.4. Inhibition of eEF-2K suppresses cyclin D1 expression and Src and activity of MAPK-ERK

To determine molecular mechanisms that are mediated by eEF-2K signaling, next we investigated the critical mediators of cell proliferation, migration and invasion, including cyclin D1, Src, and MAPK-ERK activity. Cyclin D1 that we have demonstrated to be the downstream mediator of eEF-2K signaling by promoting cell cycle [21,26] and MAPK-ERK play important roles in cell proliferation and patient survival [41,42]. Our results showed that inhibition of eEF-2K by siRNA and rottlerin treatment reduced expression of cyclin D1 and activity of ERK pathway (Fig. 5A, B). Src, a non-receptor tyrosine kinase, is one of major pathways promoting cancer cell migration/invasion and proliferation in various cancers including lung cancer [43] and considered as an important molecular target [44–46]. Inhibition of eEF-2K led to a significant reduction in Src signaling as indicated by inhibition of phosphorylation at Tyr-416 of Src in lung cancer cells (Fig. 5A, B, Supplementary figure Fig. 5).

3.5. In vivo therapeutic targeting of eEF-2K by siRNA inhibits growth of lung cancer tumor xenografts in mice

To determine the in vivo role of eEF-2K in the lung cancer tumorigenesis and validate it as a potential therapeutic target, we inhibited its expression in lung cancer tumor xenograft models (A549 and H1299) in nude mice. Tumor cells were implanted into right flank of nude mice by subcutaneous injection and about 2 weeks later eEF-2K siRNA and non-silencing control siRNA (0.3 mg/kg, i.v. from tail vein) incorporated in nanoliposomes were injected into mice once a week for four weeks. As shown in Fig. 5, mice treated with liposomal eEF-2K siRNA significantly had smaller tumors compared with those treated control siRNA in the control group in H1299 (Fig. 6A) and A549 models (Fig. 6B) ($n = 5$ animals/group, $p < 0.05$). Analysis of H1299 tumors from cells xenograft mice after 4 weeks of treatment by IHC revealed the reduced intratumoral proliferation marker Ki-67 (Fig. 6C, D).

4. Discussion

Despite recent advances in therapeutic approaches, patient survival in lung cancer has not improved substantially in the last 20 years, and only 17.4% of all lung cancer patients live 5 year after the initial diagnosis [20]. Lung carcinoma spreads primarily by extensive local invasion, early lymphatic and hematogenous routes due to highly aggressive and metastatic potential, contributing to poor clinical prognosis. Therefore, there is an urgent need for identification new molecular targets and development of novel therapeutic interventions that are able to control invasion and metastasis and tumor growth to improve survival of patients with lung cancer.

In the current study, we demonstrated for the first time that expression of eEF-2K is highly upregulated in lung cancer cells compared to nontumorigenic normal bronchial epithelium cells and associated

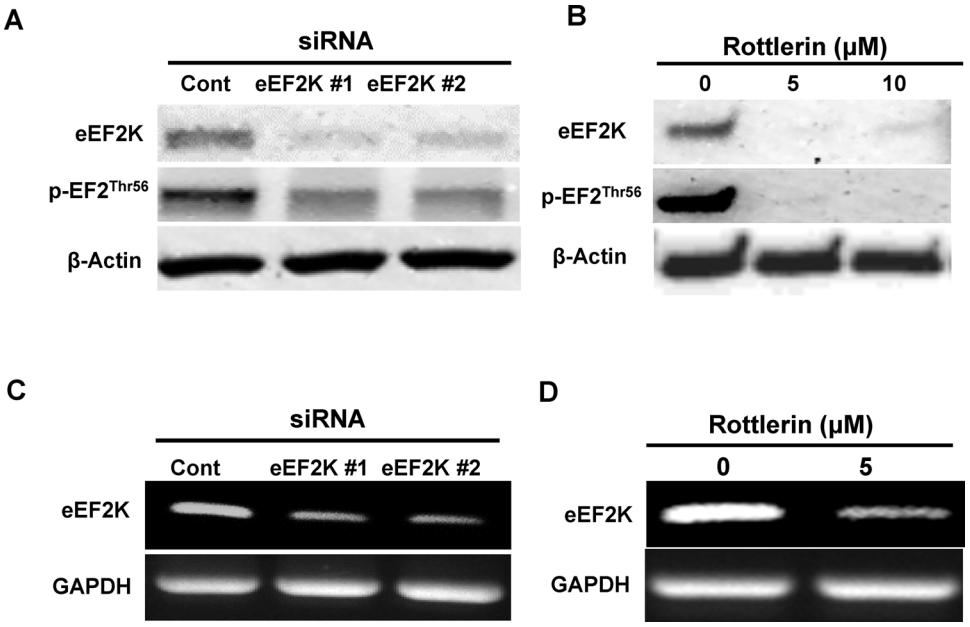

Fig. 2. The downregulation of eEF-2K expression by specific siRNAs (A) and rottlerin (B) in A549 cells. A549 cells were transfected with two different sequences of eEF-2K siRNAs (50 nM) for 72 h, or treated with rottlerin, (5 u M and 10 u M) for 48 h. eEF-2K protein expression was detected. β-actin was used as loading control western blot analysis. eEF-2K mRNA expression was also inhibited by eEF-2K siRNAs (C) or rottlerin (D). Data are represented as mean ± SD. * $p < 0.05$ vs. control condition.

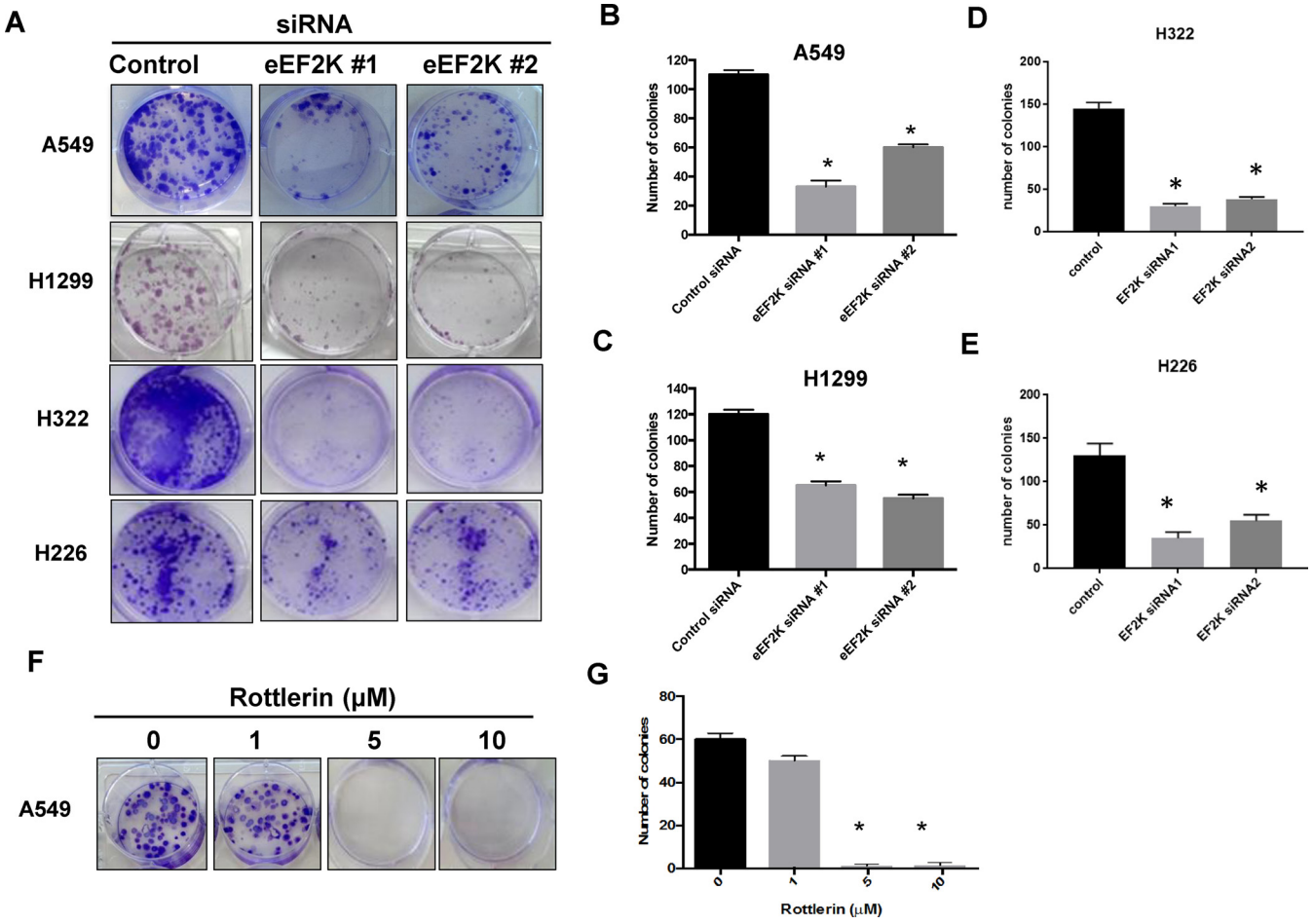

Fig. 3. Effectsof eEF-2K inhibition on lung cancer cell proliferation and colony formation. A. eEF-2K inhibition by siRNA suppresses lung cancer cell proliferation. Cells were transfected with two different sequences of eEF-2K siRNAs (50 nmol) and plated in 6 well plates (B–E). F. Rottlerin (5 u M and 10 u M) treatment inhibits cell A549 cell proliferation and colony formation. Data are represented as mean ± SD. * $p < 0.05$ vs. control condition (G). All experiments were independently performed three times.

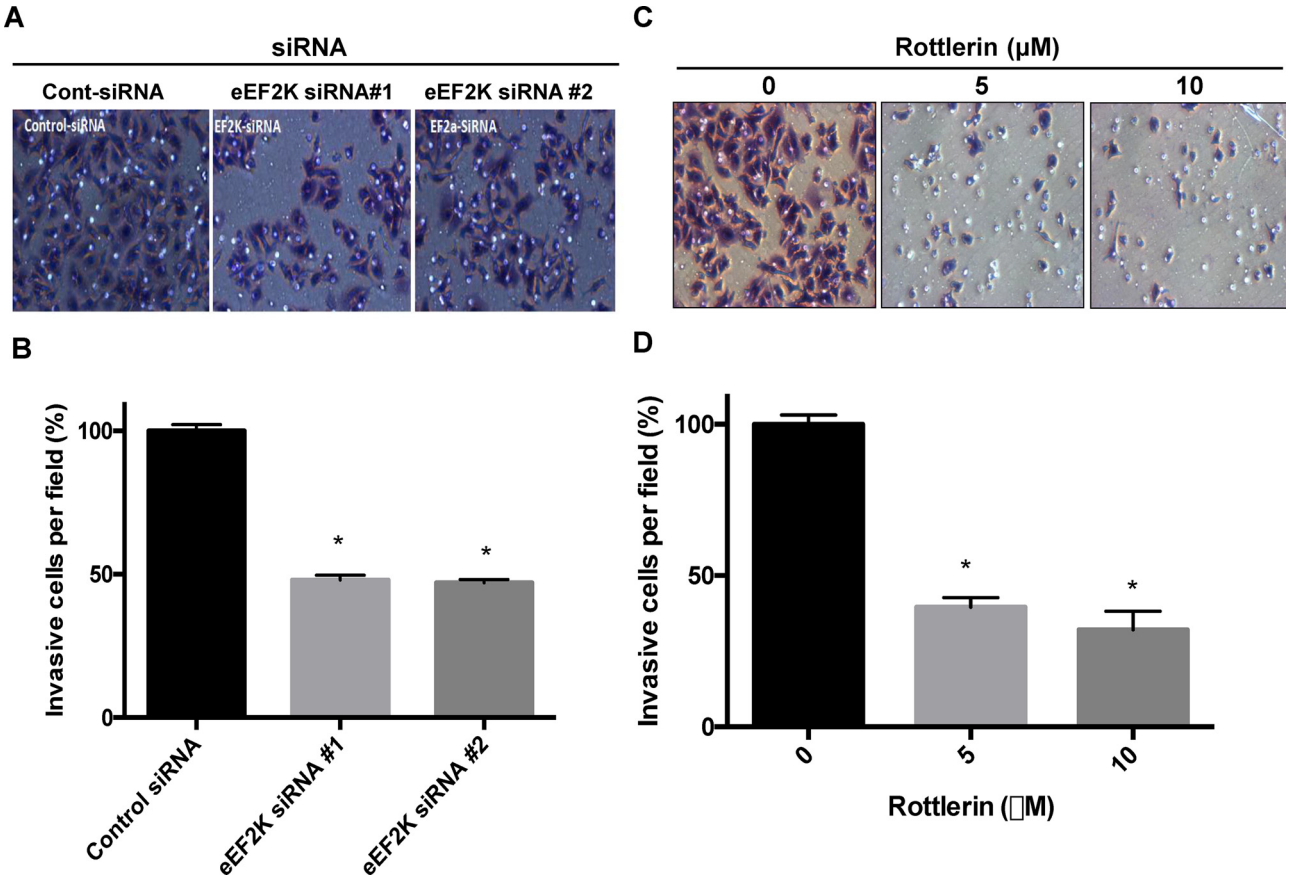

Fig. 4. Effects of eEF-2K inhibition on migration and invasion of A549 lung cancer cells. Cells were treated with indicated siRNAs for 24 h (A, B) or various rottlerin concentrations for 6 h or the vehicle (DMSO) (C, D) and seeded into matrigel invasion chambers. Twenty four hours later, the number of invaded cells was determined in a minimum of four randomly selected areas. Magnification, 100 × . The histograms show the percentages of the invaded cells. Data are expressed as mean of the percentages of invasion SD of three experiments. *$p < 0.05$ versus control cells.

with shorter patient survival and poor clinical outcome. Our findings indicated that eEF-2K plays an important role in lung cancer proliferation, invasion and tumorigenesis by promoting clinically significant pathways (Fig. 6E).

eEF-2K is an unusual α-kinase [47] that is associated with activation of AKT (protein kinase B) [48], growth factor antagonists [33], ER stressors [49], metabolic stress [50,51] and glycolytic inhibitors [52], indicating that eEF-2K can function as a survival pathway in cancer cells. Given the highly invasive phenotype of lung cancer cells, eEF-2K seems to contribute to cell migration and invasion by regulating Src signaling, which is one of the most important pathways promoting cancer cell invasion, metastasis and progression of the tumors to advance stages [53]. We found that the downregulation of eEF-2K inhibits activation of Src suggesting that eEF-2K contributes to the invasive properties of lung cancer cells. We have previously shown that eEF-2K regulates integrin-β1 expression [26]. Integrins have been shown to contribute to proliferation and invasion of many tumors and poor patient prognosis and overall survival in clinical settings and their expressions vary in lung cancer cell lines [54,55]. Although we did not focus integrin-β1 expression in the current study, we found that inhibition of eEF-2K reduced in integrin-β1 expression in lung cancer cells (data not shown), supporting our previous findings that eEF-2K

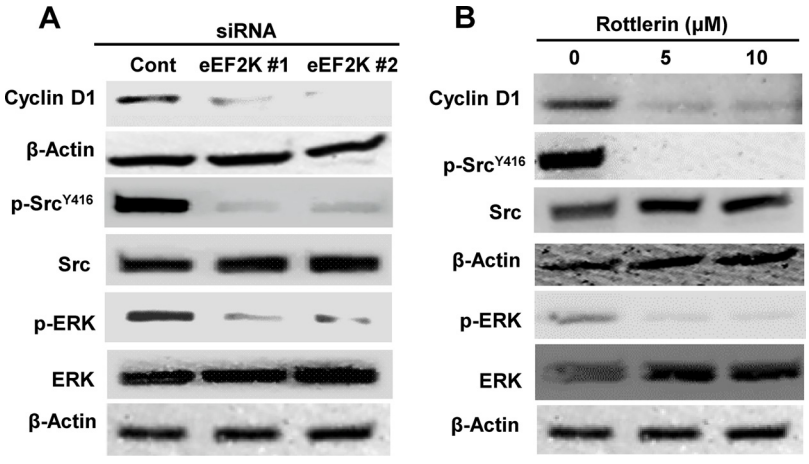

Fig. 5. Effects of eEF-2K inhibition on signaling pathways in lung cancer cells. eEF-2K siRNA (A) and rottlerin (B) treatments inhibited the expression of Cyclin D1 and the activation of Src as indicated by reduced expression of p-Src (Y416) and p-ERK-MAPK in A549 lung cancer cells. Cells were treated with 5 μM and 10 μM of rottlerin for 24 h or transfected with 50 nM of control or eEF-2K siRNA for 72 h and related protein expressions were evaluated by western blot analysis as described in the materials and methods section. β-actin was used as loading control protein expression.

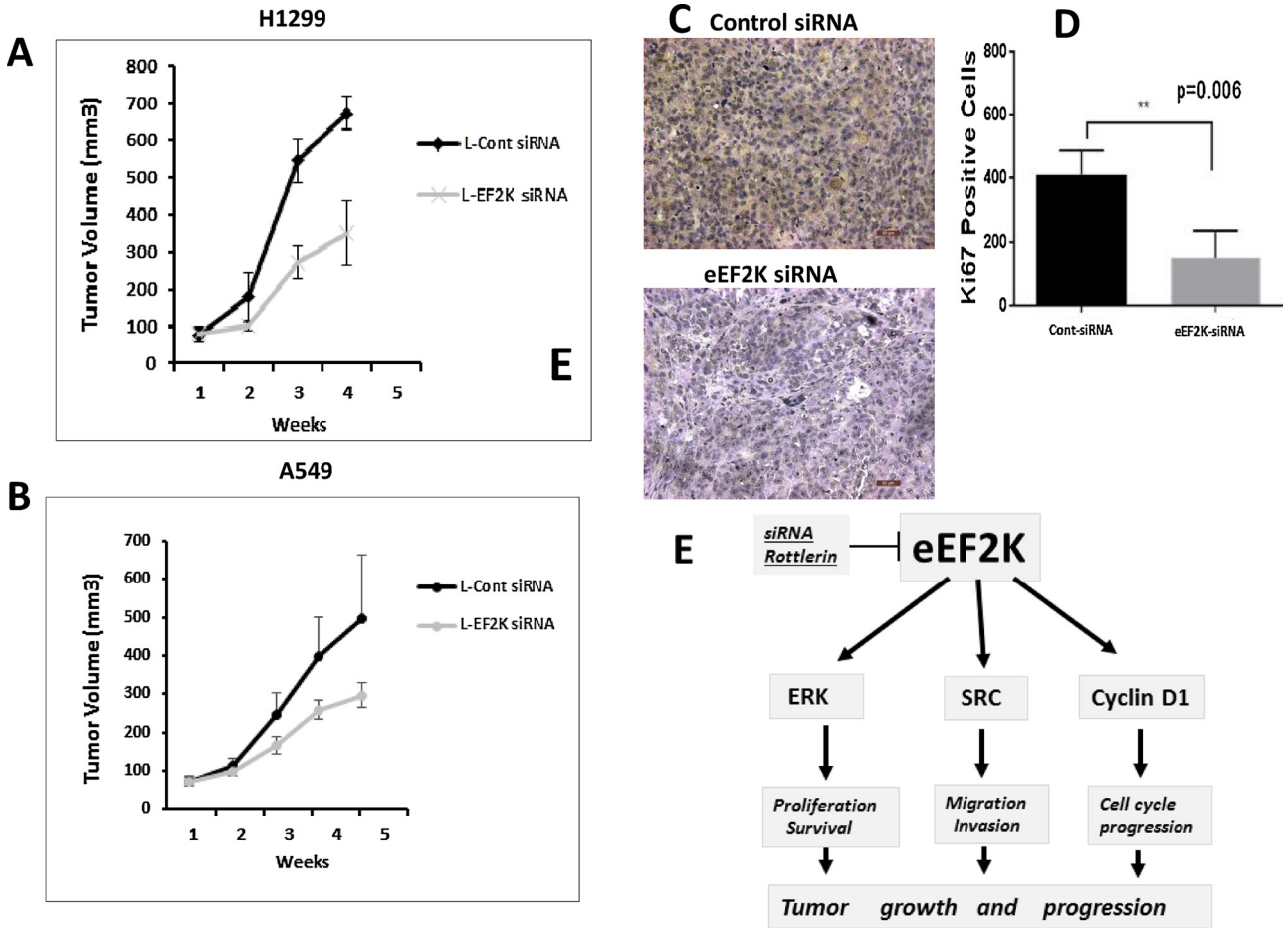

Fig. 6. In vivo systemic administration of liposomal eEF-2K siRNA inhibits tumor growth in lung cancer mouse xenograft models. H1299 and A549 cells were injected into right flank of athymic nude mice. When tumors reached 3–5 mm in diameter, mice were either treated with DMPC-based liposomal nanoparticles incorporating control siRNA or eEF-2K siRNA (0.3 mg/kg or about 8 μg/mouse, i.v once a week from tail vein, n = 5 mice/group). Tumors sizes were measured weekly. The tumor volume growth curves were shown as means ± SD of the group in H1299 (A) and A549 (B) lung cancer models. The expression of Ki-67, an intratumoral proliferation marker, was visualized by IHC in tumor xenografts in mice after 4 weeks treatments with eEF2 K siRNA (C) and quantified by densitometry as mean ± SD of the group (D). P-values were obtained with Student's *t*-test. E) The postulated molecular mechanisms of eEF-2K regulated pathways promoting tumor growth and progression in lung cancer. eEF-2K induces proliferation, cell cycle, migration and invasion though regulation of Cyclin D1, Src and MAPK-ERK signaling pathways in lung cancer.

regulates integrin-β1 expression [26]. Integrin-β1 inhibition has been shown to reduce phosphorylation of Src at Tyr 416 (activation) in lung cancer cells, indicating integrin-β1/Src axis may play an important role in tumor progression [53,56]. eEF-2K downregulation leads to a decrease in Integrin-β1, matrix metalloproteinase-2 (MMP-2), urokinase-type plasminogen activator receptor (uPAR) and mesenchymal markers, including Snail, TCF8/ZEB1, and an increase in Claudin-1 as an epithelial marker [26]. In parallel to their findings, future studies may investigate the role of eEF-2K in Integrin-β1/Src signaling axis.

eEF-2K inhibition by siRNA or rottlerin also leads to inhibition in cell proliferation and colony formation by inhibition of cyclin D1 and MAPK in lung cancer cells, suggesting that eEF-2K is involved in cell proliferation by regulating Cyclin D1 and ERK signaling. Despite our findings in lung cancer cells, there is strong evidence regarding the role of eEF-2K in cancer cell proliferation in other cancer cells [22,26]. Hamurcu et al. found that inhibition of eEF-2K and its upstream regulator Forkhead Box M1 (FOXM1) significantly inhibits cell proliferation, colony formation, migration, and invasion in triple negative breast cancer by regulating eEF-2K/cyclin D1, and MAPK-ERK signaling axis. eEF-2K was shown to be induced in cancer cells in response to multiple stresses, such as hypoxia, autophagy, nutrient starvation, and the endoplasmic reticulum stress [28]. eEF-2K is involved in cell survival by mediating autophagy in cancer cells, including lung cancer cells, requiring further investigation by future studies.

Our findings also suggest that eEF-2K expression is required for lung cancer tumorigenesis and could serve as a therapeutic target in lung cancer patients who have upregulated eEF-2K expression. Overall, studies suggest that eEF-2K plays an important role in regulation of the cell proliferation, survival, migration/invasion and tumor growth of lung cancer cells by regulating key cellular pathways and demonstrate the potential of molecular targeting of eEF-2K axis as a novel strategy in treatment of lung cancer and other cancer types.

Conflict of statement

The authors declare that they have no conflict of interests.

Acknowledgements

This study was supported by a funding from Scientific and Technological Research Council of Turkey (TUBITAK) and funding from non-coding RNA center at MD Anderson cancer Center.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

online version, at doi:https://doi.org/10.1016/j.lungcan.2018.07.027.

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