

miR-34c-3p functions as a tumour suppressor by inhibiting eIF4E expression in non-small cell lung cancer

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Abstract

Objectives: MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression and mediate diverse physiological processes. In this study, we investigated functions of miRNA miR-34c-3p in non-small cell lung cancer (NSCLC).

Materials and methods: miR-34c-3p expression was evaluated by qPCR. Cell viability was examined by MTT and proliferation by cell cycle analysis. Cell migration and invasion were tested using Transwells with/without Matrigel coating. Western blot analysis was performed for eIF4E, c-Myc, Cyclin D1, survivin and Mcl-1 protein expression.

Results: miR-34c-3p expression was significantly reduced in tissues and serum samples from NSCLC patients and in NSCLC cell lines A549, H460, H23, H157 and H1299. Overexpression of miR-34c-3p in A549 and H157 cells reduced cell proliferation, migration and invasion, whereas transfection with miR-34c-3p inhibitor (miR-34c-3p-in) produced opposite effects. Target analysis using algorithms miRanda, TargetScan and DIANA identified eIF4E as a potential target of miR-34c-3p. Luciferase assay using the eIF4E 3'-UTR reporter carrying a putative miR-34c-3p target sequence revealed eIF4E to be a specific target of miR-34c-3p. Overexpression of miR-34c-3p in NSCLC cell lines led to significant reduction in mRNA and protein levels of eIF4E,

whereas inhibition of miR-34c-3p resulted in significant increase in eIF4E protein levels, confirming eIF4E to be a direct target of miR-34c-3p in NSCLC. Overexpression of eIF4E in A549 cells promoted cell proliferation, migration and invasion, which were partially reversed by miR-34c-3p.

Conclusion: miR-34c-3p directly targeted eIF4E and reduced miR-34c-3p expression in NSCLC, promoting cell cycle progression, proliferation, migration and invasion.

Abbreviations

4E-BP1: 4E binding protein 1

Akt: protein kinase B

EGFR: epidermal growth factor receptor

eIF4E: eukaryotic translation initiation factor 4E

MAP kinase: mitogen-activated protein kinase

miRNA: microRNA

Mnk1: mitogen-activated kinase interacting kinase

mTOR: mammalian target of rapamycin

NSCLC: non-small cell lung cancer

PI3K: phosphoinositide-3-kinase

siRNA: small interfering RNA

UTR: untranslated region

VEGFR: vascular endothelial growth factor receptor

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Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs (20–24 nucleotides) that post-transcriptionally

regulate gene expression and play key roles in diverse biological processes, including development, proliferation, differentiation and apoptosis(1). miRNAs bind to complementary sequences at 3'-untranslated regions (UTR) of target mRNAs resulting either in mRNA degradation or inhibition of translation (1,2). To date, approximately 2000 miRNAs have been identified in the human genome and this number is rapidly increasing (3). More than 60% of the protein-coding genes in humans are estimated to include an miRNA target-binding site in their 3'-UTR region. As they control gene expression, miRNAs have been extensively investigated in cancer research as therapeutic targets and as biomarkers (4–7). miR-34c-3p is one of the mature miRNAs of miR-34c. It can inhibit glioma cell proliferation and induce apoptosis (8). miR-34c-3p has been found to be down-regulated in NSCLC, but its function in NSCLC tumourigenesis still remains unknown (9,10).

Lung cancer is the most common type of malignancy, worldwide. In 2014, an estimated 160,000 deaths in the United States alone were due to lung cancer, accounting for 20% of all cancer-related deaths (11). Approximately, 85% of lung cancers are classified as non-small cell carcinomas (NSCLC). Mutations or defects in several cellular kinase-signalling pathways (PI3K/mTOR/Akt, EGFR, LKB, c-MET), and genetic and epigenetic alterations have been shown to be associated with development and progression of lung cancer (12). Thus, kinases have been major targets for therapeutic development in this area. However, existing therapeutics (including recently FDA-approved EGFR inhibitor erlotinob and the VEGFR inhibitor bevacizumab) are not sufficiently effective. This is because of heterogeneity of lung cancer, which involves multiple mechanisms. Thus, improved understanding of the pathways contributing to its pathogenesis are necessary for development of effective therapeutics strategies.

Elevated expression of eukaryotic translation initiation factor 4E (eIF4E) has been found in several tumour types, including NSCLS (13–15). eIF4E recognizes and binds the 5' cap structure of an mRNA and delivers it to the eIF4F complex to enable translation (16). It has been found that expression and activity of eIF4E are directly correlated with brief survival of patients with NSCLS (17). eIF4E activity is regulated transcriptionally by c-myc (11), through phosphorylation by MAP kinase-interacting kinase Mnk1(18), and by interaction with translational repressor 4E-binding proteins (4E-BP) (19). Overexpression of phospho-eIF4E has been shown to promote cell proliferation and metastasis through the Akt pathway (20). Interfering with eIF4E expression using siRNA has been found to inhibit NSCLC cell population growth and invasion, and Mnk inhibitors have

been found to block tumour extension in experimental models of lung cancer (13), (18).

Accumulating evidence supports the notion that initiation of translation is one of the major targets of miRNAs. miRNAs have been shown to block assembly of the eIF4G complex in humans and in drosophila(21,22). Although studies have found aberrant expression of several miRNAs, including miR-221, miR-222, miR-21, miR-205, miR125, miR143, miR145, miR96, miR34 and miR30b in NSCLC (23–27), the precise nature of miRNAs that may target and regulate function of eIF4E has not been well studied. Here, we show that miR-34c-3p directly targets eIF4E expression and represses NSCLC cell proliferation, migration and invasion.

Materials and methods

Tissue samples

This study was approved by the Ethics Committee of Harbin Medical University. All NSCLC tissue samples and paired normal lung tissues were collected *via* surgical resection from patients diagnosed between 2012 and 2013 at the Harbin Medical University Cancer Hospital Department of Chest Surgery. Immediately after collection, the tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until use. Both tumour and normal samples were confirmed by pathological examination. Written informed consent was obtained from all the participants.

Cell lines and culture

Human lung cancer cell lines A549, H460, H157, H1299 and H23, and a normal diploid human lung fibroblast cell line (MRC5), were obtained from and maintained as recommended by the American Type Culture Collection (ATCC, Manassas, VA, USA). A549 and MRC-5 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA), and H460, H157, H1299 and H23 were grown in RPMI-1640 (Life Technologies). In each case, medium was supplemented with 10% foetal bovine serum (Life Technologies), 100 U/ml penicillin and 100 U/ml streptomycin. All cells were maintained at 37°C in a humidified incubator containing 5% CO_2 .

Quantitative real-time PCR

Total RNA from tissue samples and cultured NSCLC cells was extracted using TRIzol (Life Technologies) and reverse transcribed using a cDNA Library Construction Kit (Takara, Dalian, China). TaqMan quantitative

real-time PCR (qPCR) measurement of miR-34c-3p was performed on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using a microRNA assay kit (Applied Biosystems); U6 snRNA was used for normalization (28). Real-time PCR detection of eIF4E was performed using SYBR Green Master Mix (Applied Biosystems). Primers for eIF4E were: forward, 5'-TACTAAGAGCGGCTCCA CCAC-3', reverse 5'-TCGATTGCTTGACGCAGTCT CC-3'; For GAPDH, the primers were: forward, 5'-TG CACCACCAACTGCTTAGC-3', reverse 5'-GGCATGG ACTGTGGTCATGAG-3'.

Transfection

miR-34c-3p, miR-NC (negative control), miR-34c-3p-in (miR-34c-3p inhibitor) and miR-in-NC (negative control for miR-34c-3p-in) were obtained from IBSBIO (Shanghai, China) and diluted to 10 μ M concentration in nuclease-free water. mRNAs were transfected alone or in combination, into NSCLC cells at final total concentration of 15 nM, using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) and subsequent measurements performed 48 h later.

MTT assay

Cell viability was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assay. Transfected A549 and H157 cells were seeded in 96-well plates at 3×10^3 cells per well. Cells were allowed to grow for 0 to 4 days. MTT (Promega, Madison, WI, USA) solution was then added to each well and cells were incubated at 37 °C for 4 h. Absorbance was measured at 550 nm using a scanning multi-well spectrophotometer (Biotek, Winooski, VT, USA).

Cell cycle analysis

The NSCLC cells (1×10^6) were fixed in ice-cold 70% ethanol and stored at 4 °C until analysed. They were washed twice in cold PBS and incubated in 50 μ g/ml propidium iodide (PI) and 0.1 mg/ml RNase A in PBS at room temperature in dark for 1 h. The cell cycle was analysed using a flow cytometer (BD Biosciences, San Jose, CA, USA) and FLOWJO software (Treestar, Ashland, OR, USA).

Cell migration and invasion assays

In vitro cell migration and invasion assays were performed using Transwell chambers. Transfected cells (2×10^5) in 100 μ l DMEM supplemented with 1%

FBS were seeded in tops of chambers, 8 μ m pore size Transwells (PengBo Biotech, Changsha, China). For invasion assays, cells were plated in Transwells pre-coated with Matrigel (BD Biosciences). Inserts of chambers were deposited on 24-well plates containing 400 μ l DMEM supplemented with 10% FBS in the lower chamber. After 12 h incubation for migration and 24 h for invasion, non-migrating cells were removed from upper chambers. Migrated cells in lower chambers were stained with calcein AM (Life Technologies) or coomassie brilliant blue (Sigma Aldrich, St. Louis, MO, USA). Cells in six random fields were counted per chamber.

Cloning and transfection

Full-length eIF4E 3'-UTR was cloned into pMIR-reporter luciferase vector (Life Technologies). Mutations in miR-34c-3p binding sequence of the 3'-UTR of eIF4E were introduced using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) and were verified by sequencing. The eIF4E fragment lacking 3'-UTR was amplified from cDNA of A549 cells and subcloned into pcDNA3.1 (Life Technologies). Transfection of plasmid DNA was performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

Luciferase assay

Cells (5×10^4) were plated in 24-well plates overnight. miRNA mimics or inhibitors were co-transfected with wild-type or mutant pMIR-eIF4E 3'-UTR and Renilla luciferase plasmid, into cells using Lipofectamine 2000 reagent. Luciferase activities were measured with dual-luciferase reporter assays (Promega) 48 h after transfection. Luciferase activity was normalized to Renilla activity.

Western blot analysis

Cells were lysed using ice-cold RIPA buffer containing protease/phosphatase inhibitor cocktail (Boster, Wuhan, China) and protein concentration was measured using a BCA protein assay kit (Boster). Proteins in the lysate were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA, USA) which were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20 for 1 h at room temperature, and then incubated overnight at 4 °C with primary antibody against eIF4E, c-Myc, Cyclin D1, Survivin, Mcl-1 or GAPDH (Santa Cruz Biotechnologies, Dallas, TX, USA). After washing,

membranes were incubated with HRP-conjugated secondary antibodies for 1 h and bound antibodies were visualized using enhanced chemiluminescence substrate (Booster).

Statistical analysis

Statistical analyses were performed using the SPSS 19.0 software package (SPSS, Chicago, IL, USA). One-way ANOVA was used to investigate differences between groups. For all analyses, P -value of less than 0.05 was considered statistically significant. All the experiments were performed in triplicate and data are presented as mean \pm SD.

Results

Reduced miR-34c-3p expression in NSCLC tissues, patients' serum and NSCLC cell lines

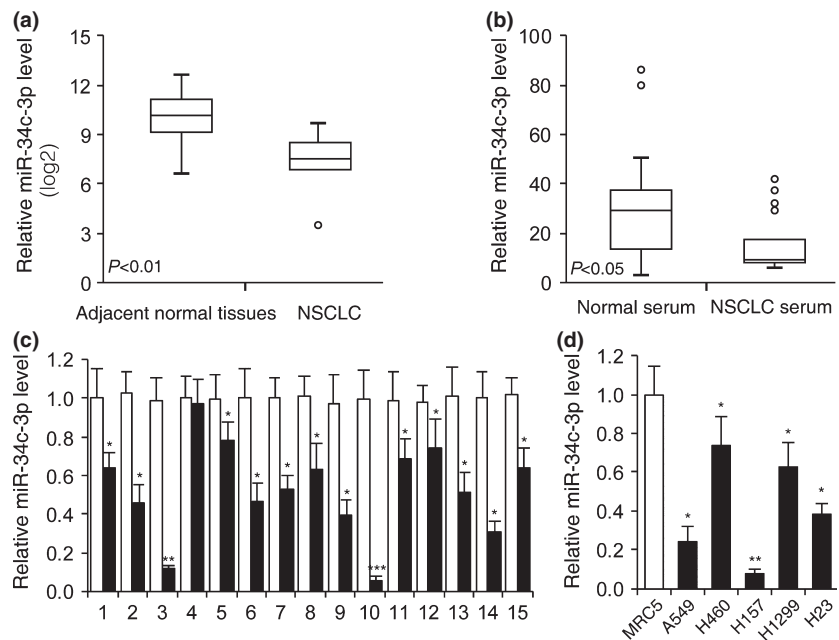
To investigate expression of miR-34c-3p in NSCLC, we searched the GSE29248 microarray database (23). The results indicated that miR-34c-3p expression was significantly lower in NSCLC tissue and serum samples of NSCLC patients than those of paired adjacent normal lung tissues ($P < 0.01$, Fig. 1a) and serum samples from healthy controls ($P < 0.05$, Fig. 1b) respectively. To further confirm that miR-34c-3p expression was reduced in NSCLC, we analysed tissue samples collected from 15 patients with NSCLC, and found that miR-34c-3p was significantly lower in 14 of the 15 samples (Fig. 1c).

We also analysed miR-34c-3p expression in five NSCLC cell lines (A549, H460, H23, H157 and H1299) and in normal lung fibroblast cell line MRC5. As shown in Fig. 1d, miR-34c-3p expression was lower in all five NSCLC cell lines than in MRC5. These results suggest that miR-34c-3p expression was down-regulated in NSCLC cells, clinical tissue samples and patient's serum.

miR-34c-3p inhibited NSCLC cell proliferation, migration and invasion

To examine the role of miR-34c-3p in development of NSCLC, A549 and H157 cells were transfected with equal amounts of miR-NC (miRNA negative control), miR-34c-3p, miR-in-NC (miRNA inhibitor negative control) or miR-34c-3p-in (miR-34c-3p inhibitor). Expression of miR-34c-3p was significantly increased by miR-34c-3p transfection (Fig. 2a), whereas miR-34c-3p-in transfection reduced endogenous miR-34c-3p expression in both A549 and H157 cells (Fig. 2b). Results of the cell proliferation assays revealed that both A549 and H157 cells, when transfected with miR-34c-3p, proliferated significantly slower than those transfected with miR-NC (Fig. 2c). In contrast, cells transfected with miR-34c-3p-in proliferated significantly faster than miR-in-NC-transfected cells (Fig. 2d). Results of cell cycle analysis revealed that overexpression of miR-34c-3p led to approximately 15% increase in number of cells in G0/G1 phase (Fig. 2e), whereas inhibition of miR-34c-3p with miR-34c-3p-in promoted

Figure 1. Expression of miR-34c-3p was down-regulated in NSCLC tissues, patients' serum and cell lines. (a) Relative miR-34c-3p expression in NSCLC tissues compared to normal tissues (GSE29248, $P < 0.01$, $n = 6$ of NSCLC tissues and adjacent normal tissues). (b) Relative miR-34c-3p expression in NSCLC patients' serum compared to normal people's serum (GSE17681, $P < 0.05$, $n = 17$ NSCLC tissues, $n = 19$ control samples). (c) Relative miR-34c-3p expression in 15 NSCLC tissues compared to adjacent normal tissues. (d) Relative miR-34c-3p expression in five NSCLC cell lines compared to normal lung fibroblast cell line MRC5 cells. $n = 4$. miR-34c-3p expression was normalized to U6 levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



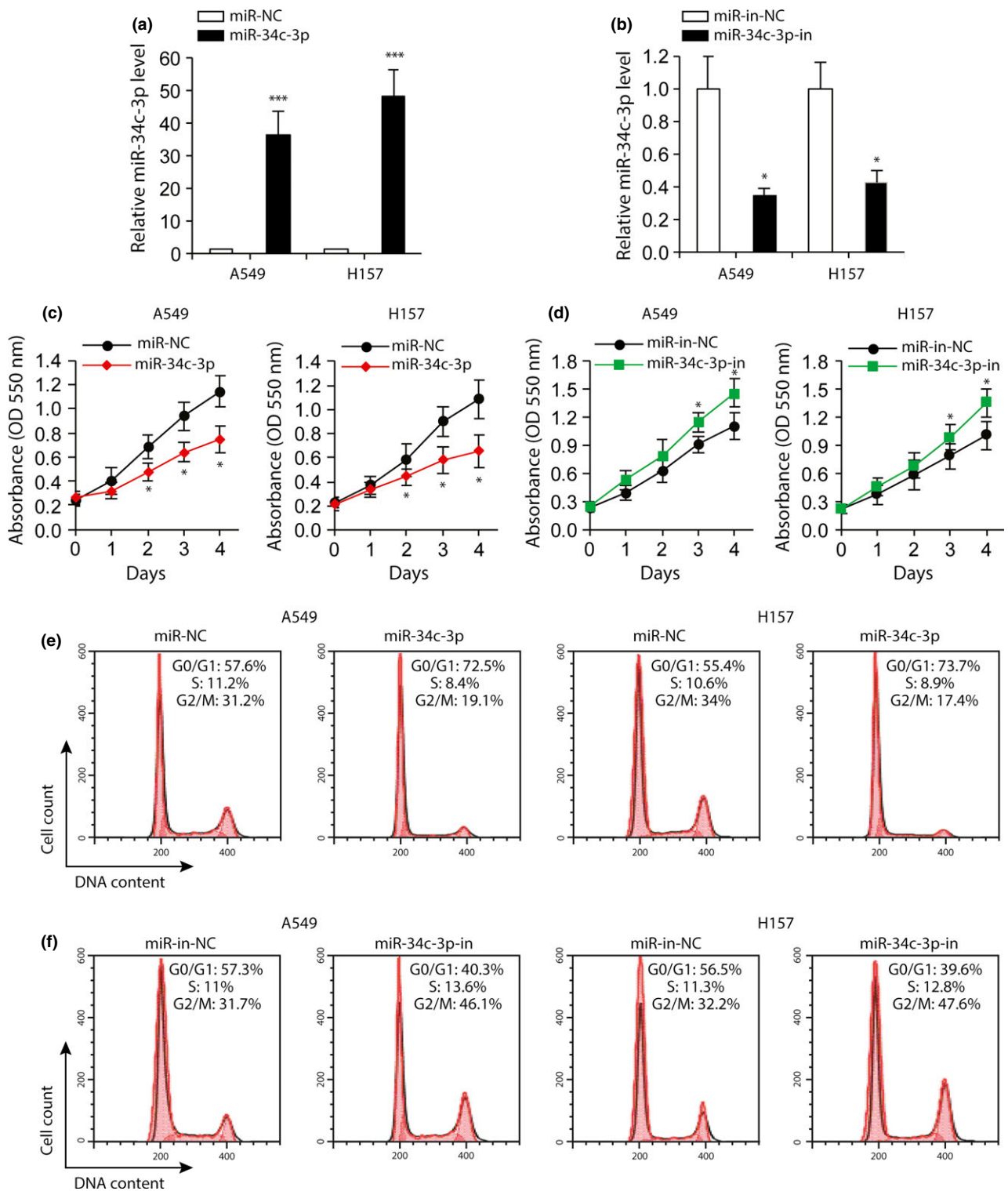


Figure 2. Effects of miR-34c-3p and miR-34c-3p-in on NSCLC cell proliferation. (a) miR-NC (negative control) or miR-34c-3p was transfected into A549 and H157 cells. Relative expression of miR-34c-3p was accessed by RT-PCR after 48 h of transfection. $n = 3$. (b) Relative miR-34c-3p levels in A549 and H157 cells following 48 h transfection with miR-in-NC or miR-34c-3p-in. (c and d) MTT cell viability assay of A549 and H157 cells transfected with miR-NC or miR-34c-3p (c), miR-in-NC or anti-miR-34c-3p (d) at indicated time points. $n = 5$. (e and f) Cell cycle analysis of A549 and H157 cells transfected with miR-NC or miR-34c-3p (e), miR-in-NC or miR-34c-3p-in (f) 2 days after transfection. $n = 4$. * $P < 0.05$, *** $P < 0.001$ compared to miR-NC- or miR-in-NC-transfected cells.

cell cycle progression with increased number of cells in S and G2/M phases (Fig. 2f).

To examine whether miR-34c-3p could regulate NSCLC cell migration and invasion, we performed a transwell assay. Overexpression of miR-34c-3p suppressed A549 and H157 cell migration (Fig. 3a), whereas inhibition of miR-34c-3p promoted migration (Fig. 3b). Furthermore, overexpression of miR-34c-3p inhibited NSCLC cell invasion (Fig. 3c), whereas miR-34c-3p-in significantly enhanced it (Fig. 3d). Taken together, these results suggest that miR-34c-3p inhibited NSCLC cell proliferation, migration and invasion.

miR-34c-3p directly targeted eIF4E in NSCLC cells

To understand molecular mechanisms by which miR-34c-3p would inhibit NSCLC cell proliferation, migration and invasion, we searched for miR-34c-3p targets

using miRanda, TargetScan and DIANA tools (29–31.) and results identified eIF4E, as a putative target of miR-34c-3p predicted in 19 vertebrate sequences (Fig. 4a). To determine whether eIF4E was a direct target of miR-34c-3p, we cloned wild-type and mutant eIF4E 3'-UTR (mutations in miR-34c-3p binding sequence) into a luciferase reporter plasmid. A549 and H157 cells co-transfected with plasmid containing wild-type eIF4E 3'-UTR and miR-34c-3p had significantly less luciferase activity than their controls (Fig. 4b). Mutation of the potential miR-34c-3p binding sites in the eIF4E 3'-UTR abolished this effect (Fig. 4b). In contrast, miR-34c-3p-in promoted eIF4E 3'-UTR wild-type luciferase activity, which was abrogated by mutating the tentative miR-34c-3p binding sites in eIF4E (Fig. 4c). Quantitative PCR and western blotting revealed that ectopic expression of miR-34c-3p inhibited both mRNA and protein expression of eIF4E in NSCLC cells (Fig. 4d and 4e). As

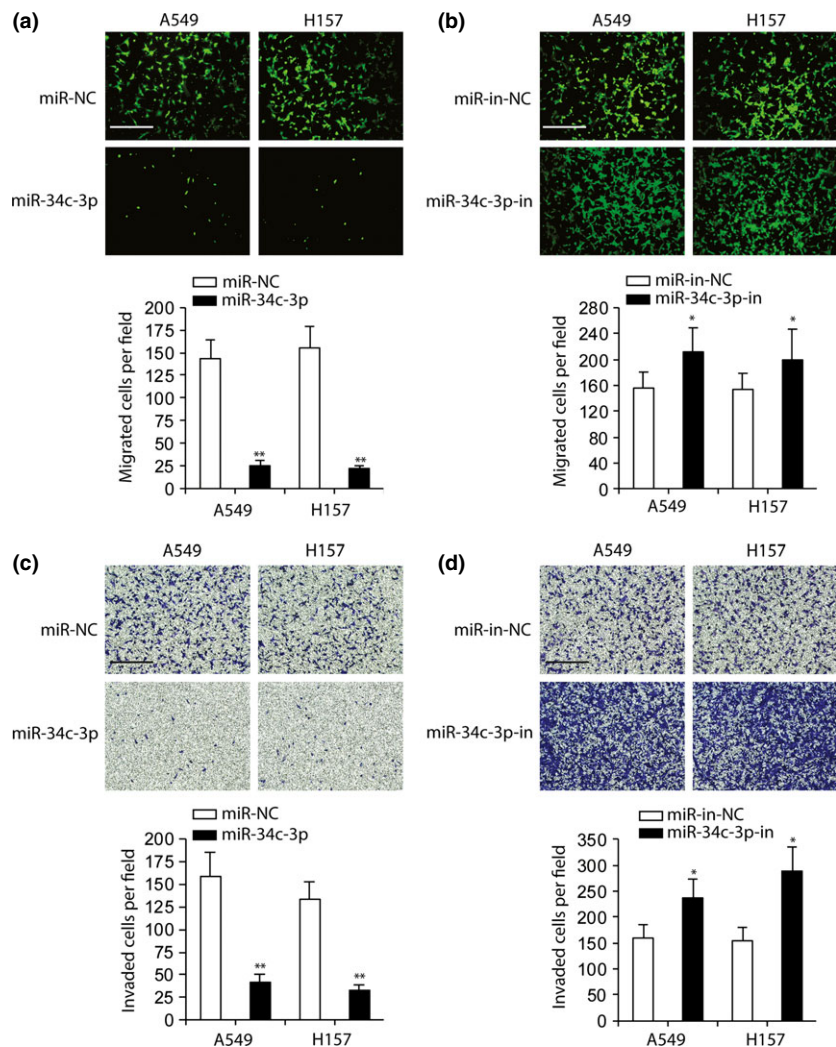


Figure 3. Inhibitory role of miR-34c-3p on NSCLC cell migration and invasion. (a–b) Migration assay in A549 and H157 cells transfected with miR-NC or miR-34c-3p (a), miR-in-NC or miR-34c-3p-in (b). Numbers of migrated cells per field were counted (bottom panel). (c and d) Invasion assay in A549 and H157 cells transfected with miR-NC or miR-34c-3p (c), miR-in-NC or miR-34c-3p-in (d); invaded cells per field were quantified (bottom panel). Scale bar: 500 μ m. $n = 4$. * $P < 0.05$, ** $P < 0.01$, compared to miR-NC- or miR-in-NC-transfected cells.

shown in Fig. 4f and 4g, inhibition of endogenous miR-34c-3p promoted eIF4E mRNA and protein expression in A549 and H157 cells. Taken together, these results suggested that miR-34c-3p directly targeted eIF4E in NSCLC cells.

miR-34c-3p/eIF4E-mediated NSCLC cell tumorigenesis

To examine whether miR-34c-3p played an inhibitory role in NSCLC progression by targeting eIF4E, we

co-transfected A549 cells with eIF4E plasmid and miR-34c-3p. Western blot analysis indicated that miR-34c-3p inhibited eIF4E overexpression (Fig. 5a). Additionally, overexpression of eIF4E significantly enhanced NSCLC cell proliferation, and partially reversed inhibition of cell proliferation caused by miR-34c-3p (Fig. 5b and 5c).

We next performed transwell assays to examine effects of eIF4E on miR-34c-3p-mediated inhibition of migration and invasion of NSCLC cells. As can be seen in Fig. 6a and 6b, transfection of A549 cells with eIF4E

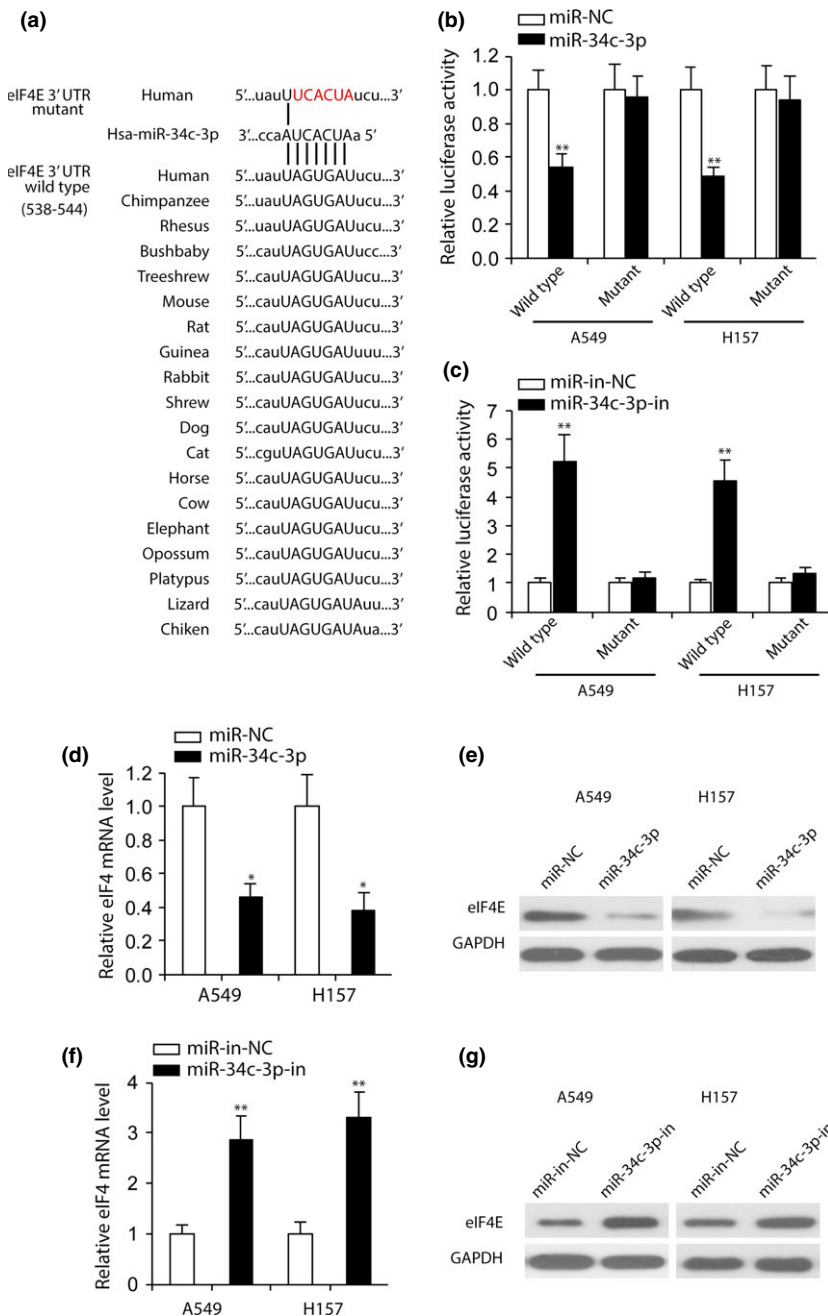


Figure 4. miR-34c-3p directly targeted eIF4E in NSCLC cells. (a) Sequences of miR-34c-3p binding sites within eIF4E 3'UTR in vertebrates. Wild-type eIF4E 3'UTR was fused into pMIR luciferase reporter plasmid. eIF4E 3'UTR mutant represents the reporter constructs containing mutated nucleotides (red). (b and c) Relative luciferase activities of plasmids carrying wild-type or mutant eIF4E 3'UTR in A549 and H157 cells co-transfected with miR-NC versus miR-34c-3p (b) and miR-in-NC versus miR-34c-3p-in (c). $n = 4$. (d and f) RT-PCR analysis of eIF4E mRNA levels in NSCLC cells transfected with miR-NC or miR-34c-3p (d), miR-in-NC or miR-34c-3p-in (f). Expression of eIF4E was normalized to GAPDH. $n = 4$. (e and g) Western blot analysis of eIF4E expression in A549 and H157 cells transfected with miR-34c-3p compared to miR-NC (e) and miR-34c-3p-in compared to miR-in-NC (g). GAPDH was used as a loading control. * $P < 0.05$, ** $P < 0.01$ compared to miR-NC- or miR-in-NC-transfected cells.

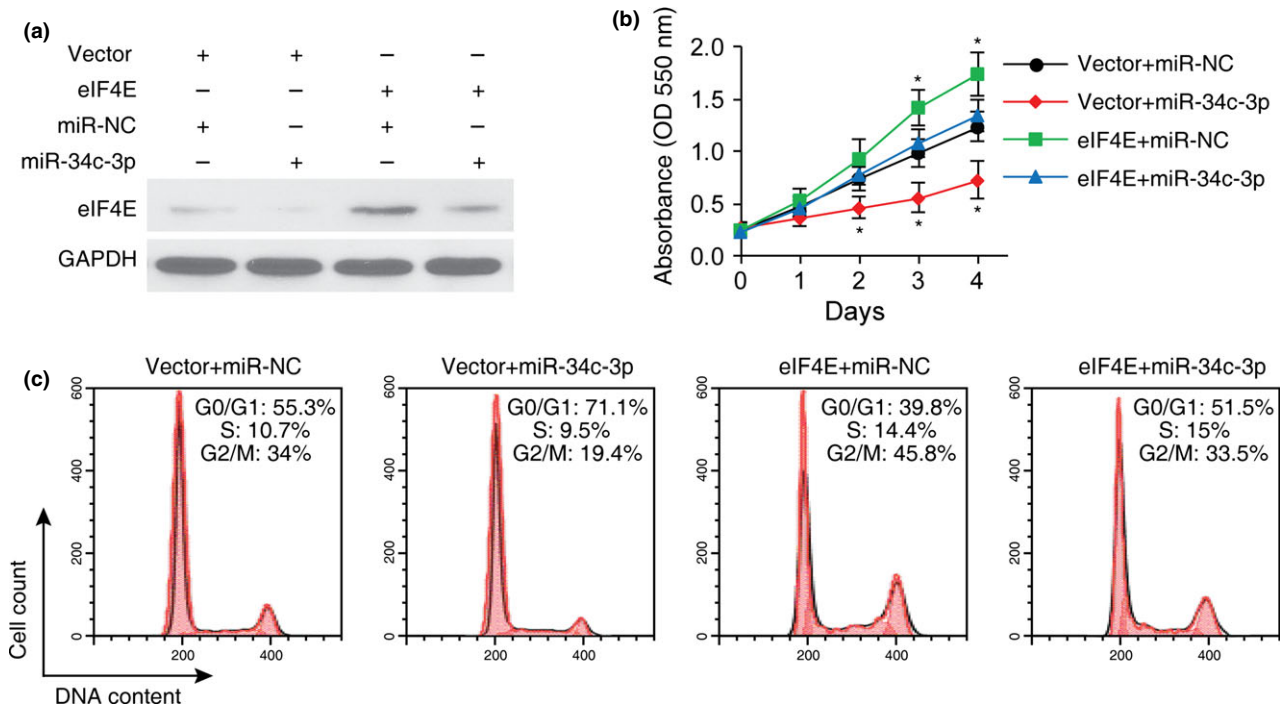


Figure 5. Overexpression of eIF4E abrogated the inhibitory role of miR-34c-3p on A549 cell proliferation. (a) Western blot analysis of eIF4E expression in A549 cells co-transfected with pcDNA3.1 vector + miR-NC, pcDNA3.1 vector + miR-34c-3p, pcDNA3.1-eIF4E + miR-NC or pcDNA3.1-eIF4E + miR-34c-3p. (b and c) MTT assay (b) and cell cycle analyses (c) of A549 cells in indicated groups. $n = 4$. * $P < 0.05$ compared to pcDNA3.1 vector + miR-NC transfected cells.

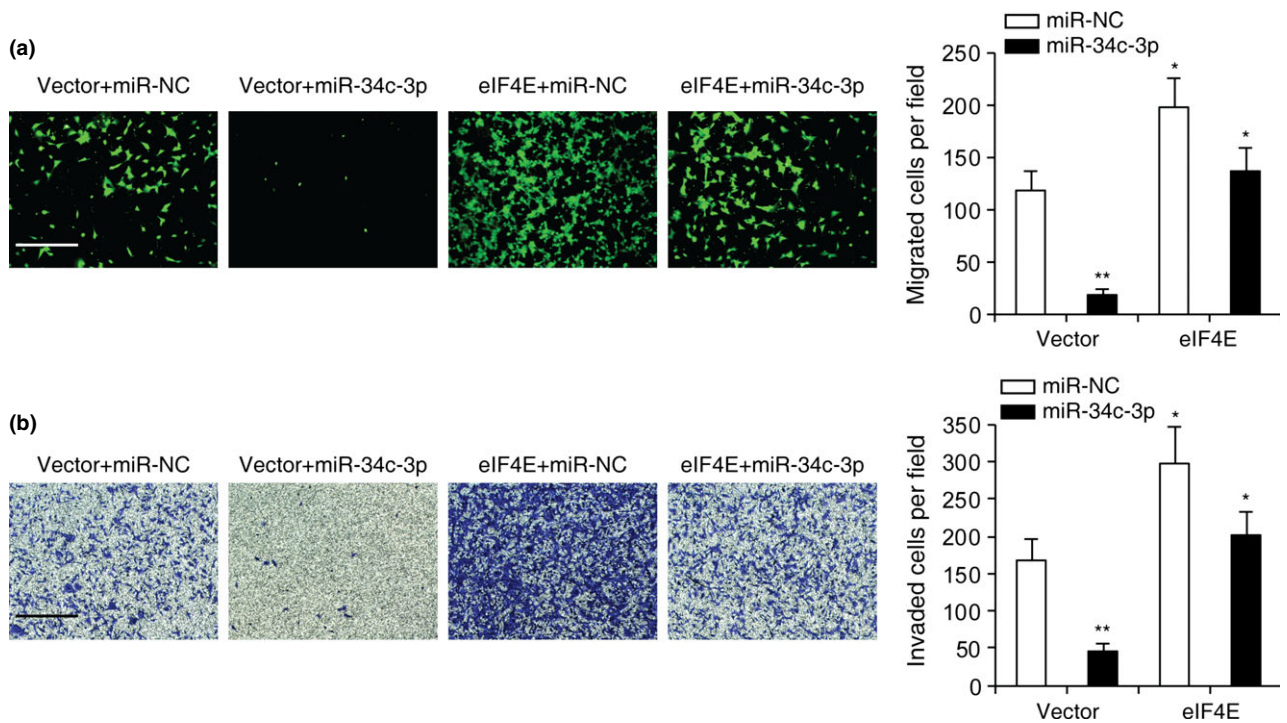


Figure 6. Overexpression of eIF4E abrogated the inhibitory role of miR-34c-3p on A549 cell migration and invasion. (a and b) Migration (a) and invasion (b) assay in A549 cells in indicated groups. Scale bar: 500 μm . $n = 4$. * $P < 0.05$, ** $P < 0.01$ compared to pcDNA3.1 vector and miR-NC transfection.

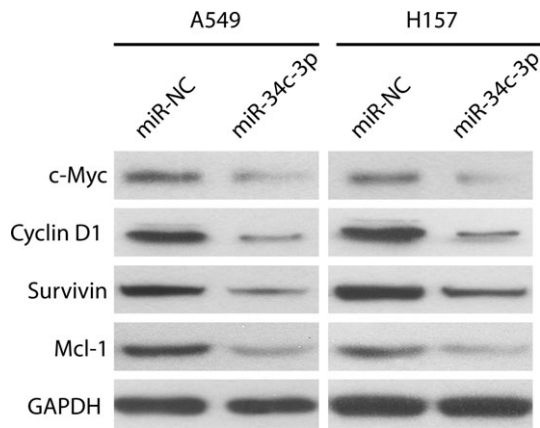


Figure 7. miR-34c-3p down-regulated eIF4E-dependent downstream gene expression. A549 and H157 cell were transfected with miR-NC or miR-34c-3p, and protein levels of c-Myc, Cyclin D1, survivin and Mcl-1 were compared using western blot analysis 48 h later. GAPDH was used as a loading control.

and miR-NC promoted migration and invasion, and eIF4E reversed inhibition of cell migration and invasion caused by miR-34c-3p. To analyse effects of miR-34c-3p on expression of genes downstream of eIF4E (c-myc, Cyclin D1, Survivin and Mcl-1) (13), we transfected miR-34c-3p into A549 and H157 cells and performed western blot analysis. Results indicated that miR-34c-3p down-regulated expression of c-myc, Cyclin D1, survivin and Mcl-1 in NSCLC cells (Fig. 7). Taken together, these results suggest that eIF4E is involved in miR-34c-3p-induced inhibition of cell migration, proliferation and invasion of NSCLC cells and that miR-34c-3p exerted anti-tumour effects likely to be by regulating expression of c-myc, Cyclin D1, survivin and Mcl-1 downstream of eIF4E.

Discussion

In this study, we found that miR-34c-3p directly targeted and inhibited expression of eIF4E and played a significant role in regulating proliferation, migration and invasion of NSCLC cells. Elevated expression of eIF4E and reduced expression of several miRNAs in NSCLC have been reported previously (12,13,32,33). However, dysregulated mechanism(s) responsible for up-regulated eIF4E expression in NSCLC had not been investigated previously.

We found that miR-34c-3p expression was significantly lower in NSCLC cells and tissue samples than in normal lung fibroblasts and paired normal tissue, respectively. Overexpression of miR-34c-3p reduced eIF4E expression and caused significant reduction in NSCLC cell proliferation, migration and invasion. Inhibition of miR-34c-3p resulted in increased eIF4E expression

along with rapid cell cycle progression, and increased cell proliferation, migration and invasion. These results suggest that miR-34c-3p functions as a tumour suppressor by limiting eIF4E expression, and that restoring expression of miR-34c-3p could be useful for treatment of NSCLC.

eIF4E is overexpressed in a variety of tumours and correlates with disease progression in NSCLC patients (14). Overexpression of eIF4E alone has been shown to be sufficient for transformation and tumorigenesis in cultured fibroblasts and epithelial cells (34–36). eIF4E is a mRNA cap-binding protein, whose activity is regulated through phosphorylation by mitogen- and stress-activated protein kinase Mnk1 or by binding to eukaryotic initiation factor 4E binding proteins (4E-BPs). Phosphorylation by Mnk1 at Ser 209 enhances eIF4E's cap-binding activity and stimulates protein synthesis (37). By binding with eIF4E, 4E-BPs negatively regulates eIF4E activity by limiting availability of eIF4E to complex with eIF4G. Phosphorylation of 4E-BP1 by mTOR releases eIF4E and increases its activity(38). Overexpression of 4E-BP1 or inhibition of its phosphorylation by rapamycin treatment inhibits cell proliferation and increases the susceptibility of the cells to undergo apoptosis.

Although regulation of eIF4E activity has been extensively studied, little is known about the mechanisms that regulate eIF4E expression. In fibroblasts, serum and growth factors increase eIF4E mRNA expression (39). Further, it has been reported that c-myc, a potent regulator of cell proliferation, increases eIF4E expression (40). eIF4E itself has been found to promote expression of c-myc, suggesting a feedback mechanism of regulation. Some studies have suggested that miRNAs regulate expression of eIF4E. Chen *et al.* have reported that miR-145 inhibited NSCLC cell proliferation by targeting the c-myc/eIF4E pathway (41). These authors observed a significant reduction of miR-145 levels in tumour tissues NSCLC cell lines. Recently, Jiang *et al.* reported that down-regulation of miR-768-3p enhanced expression of eIF4E and protein synthesis in melanoma cells (42).

It has been reported that p53 transcriptionally targets the miR-34 family to regulate c-Myc, CDK6 and c-MET expression, apoptosis and cell cycle progression (43–45). miR-34 genes are expressed in a tissue-specific manner (although miR-34a is ubiquitously expressed with highest expression in the brain), miR-34b and miR-34c are predominantly expressed in lungs. miR-34c is a precursor that generates two mature miRNAs, namely miR-34c-5p and miR-34c-3p. Reduced expression of miR-34 has been found in several tumour types, including NSCLC (9,10).

It has been shown that combination therapy using miR-34c and let-7 suppresses tumour growth in mouse models of NSCLC (46). In contrast, Catuagno *et al.* found that miR-34c-5p induces resistance to apoptosis in lung cancer (47). Although miR-34c has been identified as a tumour suppressor, its cellular targets and functions remained largely unknown.

As our analysis using miRanda, TargetScan and DIANA determined eIF4E to be a potential target for miR-34c-3p, we further analysed effects of overexpression and inhibition of miR-34c-3p on eIF4E-mediated cell proliferation and tumour progression. Overexpression of miR-34c-3p significantly reduced eIF4E mRNA and protein expression (Fig. 4d and 4e), and reduced luciferase activity in eIF4E 3'-UTR-luciferase transfected cells (Fig. 4b). Inhibition of endogenous miR-34c-3p, on the other hand, enhanced luciferase activity (Fig. 4c) and promoted eIF4E mRNA and protein expression (Fig. 4f and 4g). Consistent with reduced eIF4E expression, cell cycle progression, cell proliferation, migration and invasion were reduced in cells overexpressing of miR-34c-3p. Additionally, our results showed that when expressed together, miR34c-3p inhibited expression of eIF4E and partially reversed eIF4E-induced changes in cell cycle progression, proliferation, migration and invasion (Figs. 5 and 6). Negative control miR-NC and the inhibitor miR-34c-3p-in failed to alter effects of overexpression of eIF4E, suggesting that miR-34c-3p specifically targeted eIF4E to suppress NSCLC cell proliferation, migration and invasion. It is worth mentioning that, for results in Figs. 5 and 6, total level of eIF4E consisted of endogenous and ectopic expression of the gene. Its ectopic expression was from eIF4E plasmid which does not contain 3'UTR and cannot be targeted by miR-34c-3p. However, intact endogenous eIF4E can be regulated by miR-34-3p. Thus, the difference between eIF4E + miR-NC and eIF4E + miR-34C-3p group in terms of eIF4E protein expression (Fig. 5a), cell proliferation (Fig. 5b), cell cycle (Fig. 5c), cell migration (Fig. 6a) and cell invasion (Fig. 6b) was from down-regulation of endogenous eIF4E expression by miR-34c-3p. Results from Figs. 5 and 6 demonstrate that overexpression of eIF4E rescued the inhibitory role of miR-34-3p in NSCLC progression. In other words, miR-34c-3p inhibited NSCLC progression by targeting endogenous eIF4E which can be rescued by ectopic expression of eIF4E without 3'UTR.

Mutations in p53 are commonly found in lung cancers, with approximately 75% frequency seen in NSCLC (48). Some studies have found that transcriptional activation of the miR34 family by p53 occurs during DNA damage and apoptosis (49). Therefore, it is likely that

reduced expression of miR-34c-3p in NSCLC is a direct reflection of changes in p53 activity during tumour progression.

In summary, we found that miR-34c-3p negatively regulated eIF4E expression, and that miR-34c-3p expression was down-regulated in NSCLC. Down-regulated miR-34c-3p expression allows for aberrant eIF4E expression, which promotes oncogene expression, cell cycle progression, proliferation, migration and invasion. Our observations provide new insight into NSCLC biology and potentially a new avenue for NSCLC therapy.

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