BRITISH JOURNAL OF BIOMEDICAL SCIENCE
2019, VOL. 76, NO. 1, 17–23
https://doi.org/10.1080/09674845.2018.1520066

microRNA-935 is reduced in non-small cell lung cancer tissue, is linked to poor outcome, and acts on signal transduction mediator E2F7 and the AKT pathway

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ABSTRACT

Background: A potential role for microRNA-935 (miR-935) has been identified in several cancers but not in non-small cell lung cancer (NSCLC). We hypothesised changes in miR-935 in NSCLC, and proposed mechanisms that may further explain its role in carcinogenesis.

Methods: NSCLC tissue and nearby normal tissue was obtained from 101 patients and was probed by qRT-PCR for miR-935 expression. The role of miR-935 and a potential target (signal transduction factor E2F7) was determined in cell lines by a dual luciferase assay. The function of miR-935 was investigated through metabolic activity (MTT) and transwell migration assays. Western blot and immunocytochemical assays examined protein expression level. Growth of miR-935 transfected or untransfected cells was measured via xenograft tumour formation.

Results: miR-935 was reduced in cancer tissue and was related to lymph node metastases, tumour node metastasis status and poor prognosis (all $p < 0.02$). *In vitro*, miR-935 suppressed cell proliferation, migration and invasion in NSCLC cells through targeting E2F7. Furthermore, E2F7 was upregulated in NSCLC tissue associated with poor prognosis ($p = 0.0203$) of NSCLC patients. miR-935 suppressed the epithelial-mesenchymal transition and AKT pathways in NSCLC and inhibited the tumour growth *in vivo*.

Conclusion: Altered miR-935 in lung cancer biopsy tissue may be a diagnostic tool and could direct treatment. Involvement in carcinogenesis is implied by its suppression of the development of NSCLC via targeting E2F7 and inhibiting AKT pathway.

ARTICLE HISTORY
Received 26 July 2018
Accepted 27 August 2018

KEYWORDS
miR-935; E2F7; non-small cell lung cancer; AKT pathway

Introduction

Lung cancer is a rapidly increasing cause of morbidity and mortality, and one of the most serious malignancies in both men and women [1,2]. Non-small cell lung cancer (NSCLC) accounts for about 75–80% of lung cancers [3]. Surgery is the first and primary treatment and the only one that can cure, the 5-year survival rate being 30–44% [4]. Chemotherapy is also a treatment for NSCLC, the remission rate being 40–50% [5]. However, chemotherapy is rarely curative, but prolongs the patient's survival and improves quality of life. Therefore, finding new effective treatments is necessary for improving the survival of NSCLC patients.

MicroRNAs (miRNAs) are likely regulators of the development of a number of cancers by targeting certain genes [6]. miRNAs may also participate in the pathogenesis and tumourigenesis of NSCLC: miR-1260b promotes migration and invasion in NSCLC via targeting receptor-type tyrosine-protein phosphatase kappa [7], whilst miR-223-5p suppresses tumour growth and metastasis in NSCLC by targeting transcription factor E2F8 [8]. Recently, miR-935 has been identified as having an effect on human cancers: knockdown in a lung cancer cell line increase cell sensitivity to chemotherapy [9], it promotes cell proliferation by targeting transcription factor SOX7 in gastric cancer [10], and it suppresses the tumourigenesis of gastric signet ring cell carcinoma by targeting Notch1 expression (a transmembrane molecule with roles in intercellular signalling) [11]. Based on these studies, we speculated a role for miR-935 in NSCLC via the E2F transcription pathway, which regulates cellular differentiation, the cell cycle and cancer development [12]. This is justified as E2F7 overexpression disrupts progression of the cell cycle [13], and leads to tamoxifen resistance in breast cancer cells through regulating by miR-15a/16 [14]. E2F7 can also regulate transcription and maturation of multiple miRNAs to suppress cell proliferation [15] and may be linked to the epithelial-mesenchymal transition (EMT) and PI3K/AKT signalling pathways, identified as participants in tumour progression and metastasis [16,17].

We hypothesised low levels of miR-935 in NSCLC tissues, and that it has a role in carcinogenesis via

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E2F7, the EMT and the AKT pathway. The latter was determined in a series of *in vitro* and *in vivo* experiments.

Materials and methods

A total of 101 NSCLC tissues and adjacent normal lung samples were obtained from patients attending the First Affiliated Hospital of Jia Mu Si University after receiving written informed consent. No patient received treatment prior to the operation. Tissues were frozen in liquid nitrogen and stored at −80 °C. The study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Jia Mu Si University. The sections of lung tissues were processed by standard techniques, dewaxed, hydrated and washed twice with PBS (phosphate buffer saline) for 5 min. After blocking with 5% goat serum (diluted in PBS), sections were incubated with rabbit polyclonal anti-E2F7 antibody (1/1000, Abcam, Cambridge, MA, USA) at 37 °C for 1–2 h. Slides were washed three times with PBS for 5 min and incubated with HRP (horse radish peroxidase)-conjugated goat anti-rabbit secondary antibody at 37 °C for 1 h. After washing three times with PBS, DAB was used for colour development. The section was washed, counterstained, dehydrated and mounted. Images captured using microscopy were scored as follows. The percentage of positive cells was expressed as: 0 (negative) for <10%; 1 (weak) for 10–30%; 2 (moderate) for 31–50% and 3 (strong) for >50%. Samples were considered 'high intensity' when staining ≥20% of the cells and 'low intensity' when staining <20% of the cells. TRIzol (Invitrogen, Carlsbad, CA, USA) was used for extracting total RNA according to the standard method. Synthesis of cDNA was by the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). Quantitative RT-PCR was carried out through the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) on ABI 7500 cycler. U6 and GAPDH were used as control for miR-935 and E2F7. Their expression was calculated using the $2^{-\Delta\Delta ct}$ method.

Lung cancer cell lines H292, A549, H1299 and virally transformed BEAS-2B epithelial cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI-1640 medium with 10% foetal bovine serum (FBS) and cultured at 37 °C with 5% CO_2. The miR-935 mimic or inhibitor, miR-935 plasmid and negative control (NC) (Ribobio, Guangzhou, China) were transferred into A549 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufactures' protocols. A549 cells transfected for 48 h were cultured in 96-well plates (2×10^3/well). Cells containing miR-935 mimic or inhibitor were incubated for 24, 48, 72 and 96 h. To determine general metabolic activity, cells were supplemented with 20 µL MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solutions (Thermo Fisher Scientific, Inc, Shanghai, China) and incubated for 4 h at 37 °C. Culture supernatant was discarded and absorbance at 490 nm was detected with a spectrophotometer.

Transwell chambers (8-µm pore size membranes) were used to perform cell migration and invasion assays. The lower chamber contained 10% FBS, the upper surface with matrigel (BD Biosciences, Corning, NY, USA) was used for cell invasion. The cell migration assay was conducted without matrigel. 2×10^4 A549 cells were cultured in the upper chamber with serum-free medium and incubated at 37 °C with 5% CO_2. 24 h later, the migrated or invasive cells were fixed with methanol and stained with crystal violet. The number of migrated cells was counted manually in at least five random fields by light microscopy.

The wild or mutant type of 3′-UTR of *E2F7* was inserted into the pmirGLO luciferase vector (Promega, Madison, WI, USA) to perform luciferase reporter experiments. Wild or mutant type of 3′-UTR of *E2F7* and miR-935 mimic were transfected into A549 cells. Subsequently, the dual luciferase assay system (Promega) was used to analyse luciferase activity. To determine EMT status, protein samples were obtained using radio immunoprecipitation assay protein lysis buffer (pH 7.4), containing 20 mM sodium phosphate, 150 mM sodium chloride, 5 mM EDTA, 5 mM phenylmethylsulphonyl fluoride, 1% aprotinin, 1 µg/ml leupeptin, and 500 µM Na_3VO_4 lysis buffer, separated through a 10% SDS-PAGE and incubated with 5% non-fat milk in polyvinyllidene diflouride membranes (Millipore, Billerica, MA, USA) at room temperature. Membranes were incubated overnight at 4 °C with E-cadherin, N-cadherin, vimentin (all considered key components of EMT), AKT, E2F7 and GAPDH (as a general positive control and household marker) primary antibodies (1:1000; Abcam, USA). After washing, membranes were incubated with goat polyclonal anti-rabbit IgG secondary antibody (1:2000; Abcam, USA). Protein expression levels were measured by ECL (ECL, Pierce, Shanghai, China).

We purchased nude mice (3–5 weeks old) from the Shanghai Lab Animal Research Center (Shanghai, China). All animal experiments were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Jia Mu Si University and conducted according to the Guide for the Care and Use of Laboratory Animals published by the US NIH. 5×10^6 A549 cells transfected (as above) with pre-miR-935 plasmid or NC were injected into the right flank of four nude mice. The tumour volume was observed every 3 days. After 4 weeks, the mice were sacrificed by CO_2 asphyxiation and tumours were taken for further study.

Data were analysed by SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) and Graphpad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). The data are shown as mean with SD and analysed by Student's *t* test. The relationship between miR-935 expression and clinic-pathological features of NSCLC patients was analysed by χ^2 test. Kaplan–Meier analysis was applied to survival data, the log-rank test compared survival differences. Significant difference was defined at $p < 0.05$.

Results

miR-935 expression was markedly lower in NSCLC tissues (mean [SD] relative expression 0.33 [0.13]) compared to normal tissues (1.0 [0.17]) (Figure 1(a)). Downregulation of miR-935 was liked with tumour node metastasis (TNM) stage and lymph node metastasis (Table 1). Kaplan–Meier analysis showed that low miR-935 expression (less than median) was related to poor prognosis of NSCLC patients (Figure 1(b)). Reduced miR-935 was linked to increased expression of signal transduction marker protein E2F7 in the cytoplasm of NSCLC tissue (representative sections shown in Figure 1(c)). Protein expression intensity of E2F7 was increased (77.5% [0.43]) in NSCLC tissues compared with the adjacent normal tissues (22.5 [0.38]) (Figure 1(d)). High E2F7 expression was related to poor prognosis of NSCLC patients (Figure 1(e)).

Reduced expression of miR-935 was present in the three lung cancer cell lines (H292, A549, H1299) compared to the non-cancer cell line (BEAS-2B) (Figure 2(a)). A549 cells transfected with miR-935 plasmids expressed increased miR-935 compared to untransfected cells, whilst expression was suppressed in cells transfected with an miR-935 inhibitor ($n = 3$ experiments in each case) (Figure 2(b)). After transfection, the MTT metabolic activity assay revealed that overexpression of miR-935 repressed the proliferation of A549 cells whereas opposite effect of miR-935 downregulation was identified for cell proliferation (Figure 2(c,d)). The transwell migration assay showed that cell migration was impaired by the transfection of miR-935 mimics, but miR-935

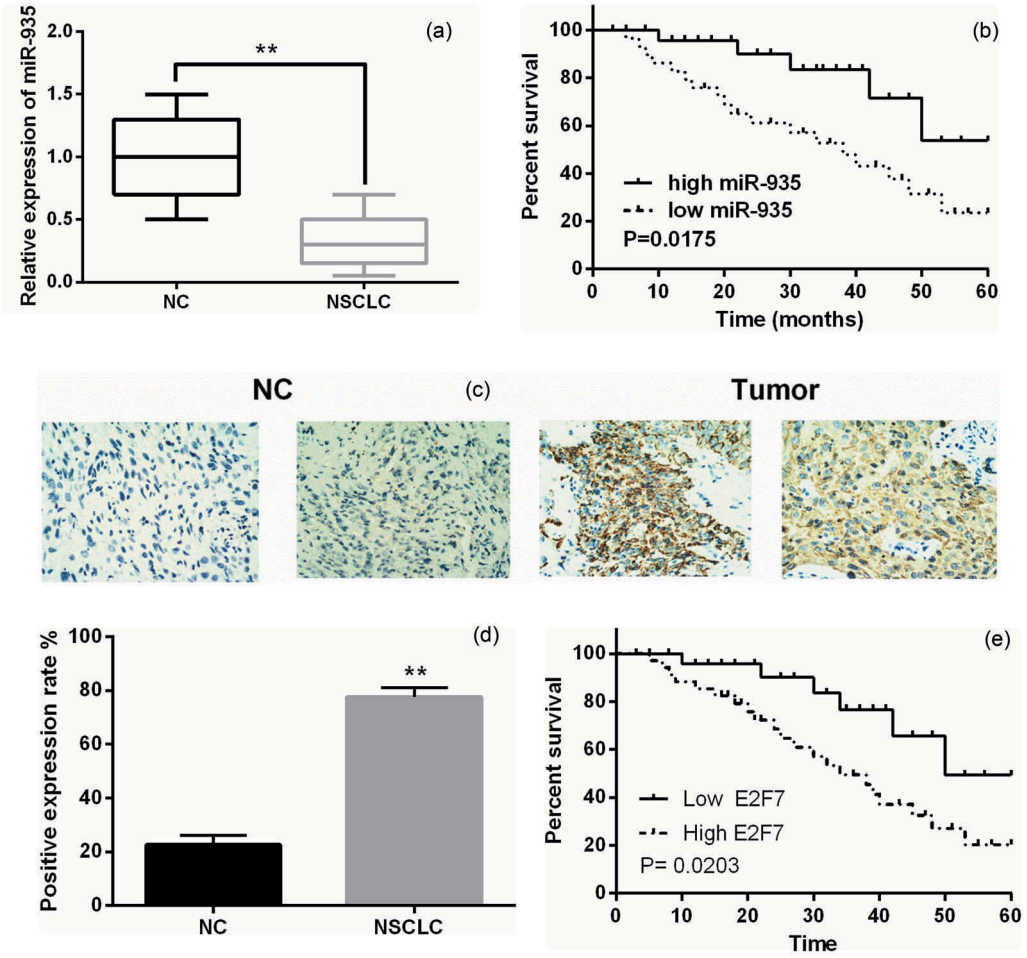

Figure 1. (a) The expressions of miR-935 in NSCLC tissues detected via qRT-PCR. (b) Lower miR-935 expression (defined by median level) was related to shorter overall survival (OS) in patients. (c, d) Protein expression of E2F7 in NSCLC tissues detected by immunohistochemistry (e) High E2F7 expression related to shorter OS. *$p < 0.05$, **$p < 0.01$.

Table 1. Relationship between miR-935 expression and their clinic-pathological characteristics of NSCLC patients.

		miR-935		
Characteristics	Cases	High	Low	*p*-Value
Age (years)				0.795
≥60	56	20	36	
<60	45	20	25	
Gender				0.263
Male	60	25	35	
Female	41	15	26	
Tumour size				0.082
<5 cm	63	28	35	
≥5 cm	38	18	20	
TNM stage				0.01
I–II	71	30	41	
III–IV	30	13	17	
Lymph node metastasis				0.007
No	75	31	44	
Yes	26	11	15	

Statistical analyses were performed by the χ^2 test.

inhibitor significantly promoted cell migration in A549 cells (Figure 2(e)). The same tendency of cell invasion was also identified in A549 cells with miR-935 mimics or miR-935 inhibitor (Figure 2(f)). Furthermore, we found that E2F7 was a target gene of miR-935 predicted by TargetScan (http://www.targetscan.org/) (Figure 2(g)). The reduction of luciferase activity was observed in A549 cells with miR-935 mimics and E2F7-wild type vector, although miR-935 mimics did not affect the luciferase activity of E2F7-Mutant (Figure 2(h)). E2F7 mRNA expression inversely correlated with miR-935 in NSCLC tissues (Figure 2(i)). mRNA expression of E2F7 was downregulated by transfecting miR-935 mimics into A549 cells (0.35 [0.13]) compared to the control group (0.95 [0.35]) (Figure 2(j)) and upregulated by transfection of miR-935 inhibitor (1.85 [0.41]) compared to the control group (0.91 [0.31]) (Figure 2(k)).

EMT western blot results were as follows. Overexpression of miR-935 suppressed N-cadherin and vimentin expressions and promoted E-cadherin expression (Figure 3(a)). Conversely, downregulation of miR-935 inhibited E-cadherin expression and enhanced N-cadherin and vimentin expression level (Figure 3(b)). There was no difference in expression of general household marker GADPH. Upregulation of miR-935 repressed the phosphorylation of AKT (i.e. p-AKT) expression in A549 cells (Figure 3(a)) whilst downregulation of miR-935 enhanced the phosphorylation of p-AKT expression (Figure 3(b)). Upregulation of miR-935 reduced the tumour volume compared to the control group (representative tumours shown in Figure 3(c)), and tumours with miR-935 stable transfection plasmid grew more slowly than those with miR-NC: at day 27 mean [SD] tumour volumes 856 [12] mm^3 vs 553 [8] mm^3 (Figure 3(d)).

Discussion

Recently, the high rates of cigarette smoking and environmental pollution have led to an increase in the incidence and mortality of lung cancer, especially NSCLC. Specific functions of miRNAs have been identified in NSCLC. For example, miR-138 inhibited cell growth, invasion, and EMT of NSCLC via SOX4/p53 feedback loop [18]. Here, we also found that overexpression of miR-935 suppressed cell growth, invasion, and EMT in NSCLC. Tang et al. reported that miR-212 expression was significantly related to poor prognostic features including positive TNM and advanced TNM stage [19]. In this study, miR-935 was also related to TNM stage and lymph node metastasis which predicted poor prognosis of NSCLC patients. miR-770 inhibits tumourigenesis and EMT by targeting JMJD6 and regulating WNT/β-catenin pathway in NSCLC [20]. MiR-935 function as a tumour-suppressor in NSCLC through targeting E2F7 and inhibiting AKT pathway.

The abnormal expression and function of miR-935 have been found in several human cancers, e.g., upregulation of miR-935 in bladder cancer [21]. However, the expression of miR-935 was decreased in NSCLC reported by previous study [22], consistent with our result. Similarly, the function of miR-935 is also different in other cancers. For example, miR-935 promotes cell proliferation and migration by targeting SOX7 in liver cancer [23]. Upregulation of miR-935 also promotes the malignant aspect of pancreatic carcinoma via targeting INPP4A [24]. Our findings were the opposite of above results. Overexpression of miR-935 inhibited the proliferation and migration of NSCLC, which may be due to the different tumour type and target genes we selected. Liu et al. found that miR-935 inhibited proliferation and invasion in osteosarcoma [25]. Compared with previous studies of miR-935, the tumour growth and EMT of NSCLC were also investigated in the present study. We found that miR-935 suppressed the growth and metastasis of NSCLC through targeting E2F7 and inhibiting EMT and AKT pathway.

In recent years, the function of E2F7 has been identified in human cancers. As a novel mammalian E2F family member, E2F7 is able to block cellular proliferation [26]. However, upregulation of E2F7 in endometrial carcinoma promotes cell proliferation [27]. Ye et al. found that miR-30a-5p inhibited gallbladder cancer cell proliferation, migration and metastasis by targeting E2F7 [28]. Consistent with these findings, we found upregulation

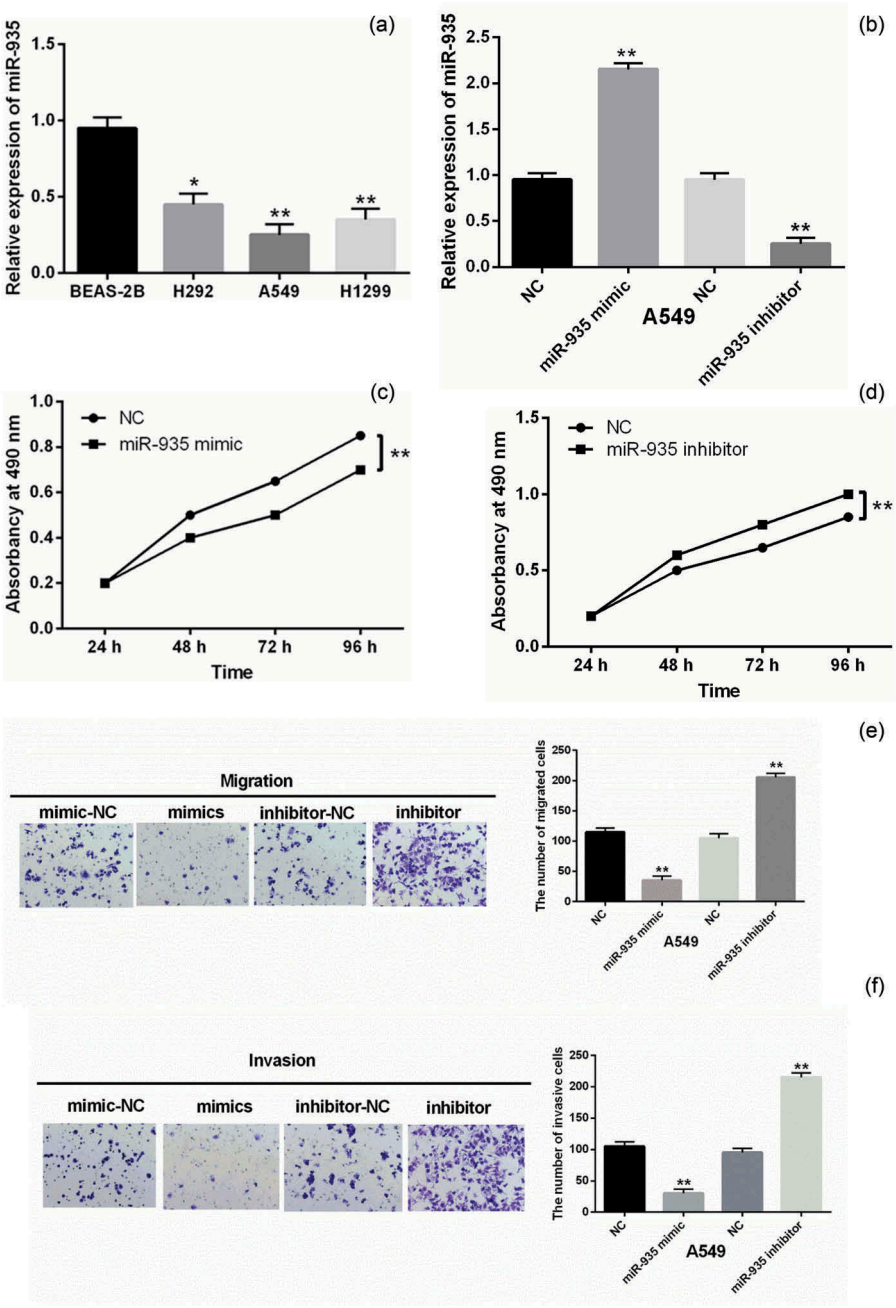

Figure 2. (a) The miR-935 expression in H292, A549 and H1299 cells and BEAS-2B cell lines. (b) The miR-935 expression was examined in A549 cells with miR-935 mimics or inhibitor via qRT-PCR. (c, d) The cell proliferation was measured in cells containing miR-935 mimics or inhibitor via MTT assay. (e, f) Cell migration and invasion analysis in cells containing miR-935 mimics or inhibitor was detected by transwell assay. (g) The binding sites of miR-935 on the 3′-UTR of E2F7 (h) Luciferase reporter assay (i) The correlation between miR-935 and E2F7. (j, k) The expression of E2F7 were observed in A549 cells containing miR-935 mimics or inhibitor *$p < 0.05$, **$p < 0.01$.

of E2F7 in NSCLC, and that miR-935 inhibited the proliferation, migration and invasion of NSCLC by targeting E2F7. E2F7 is also a direct target of other miRNAs which was negatively regulated by them, such as miR-26, miR-129, and miR-302 [29–31]. The inverse correlation between miR-935 and E2F7 was also observed in NSCLC tissues. In addition, the AKT signalling pathway had been shown to be activated by miRNAs in NSCLC, such as miR-92a [32], whilst miR-30a suppressed cell proliferation in NSCLC through PI3K/AKT signalling pathway [33]. Similarly, miR-935 repressed cell proliferation through the AKT pathway in NSCLC.

In conclusion, downregulation of miR-935 is present in NSCLC and is related to poor prognosis. Moreover, miR-935 suppressed the development of NSCLC via targeting E2F7, and suppressed the metastasis and proliferation through inhibiting EMT and AKT pathway. Our work represents an advance in biomedical science because it points to the value of the measurement of miR-935 in the diagnosis of NSCLC, and potential as a therapeutic target.

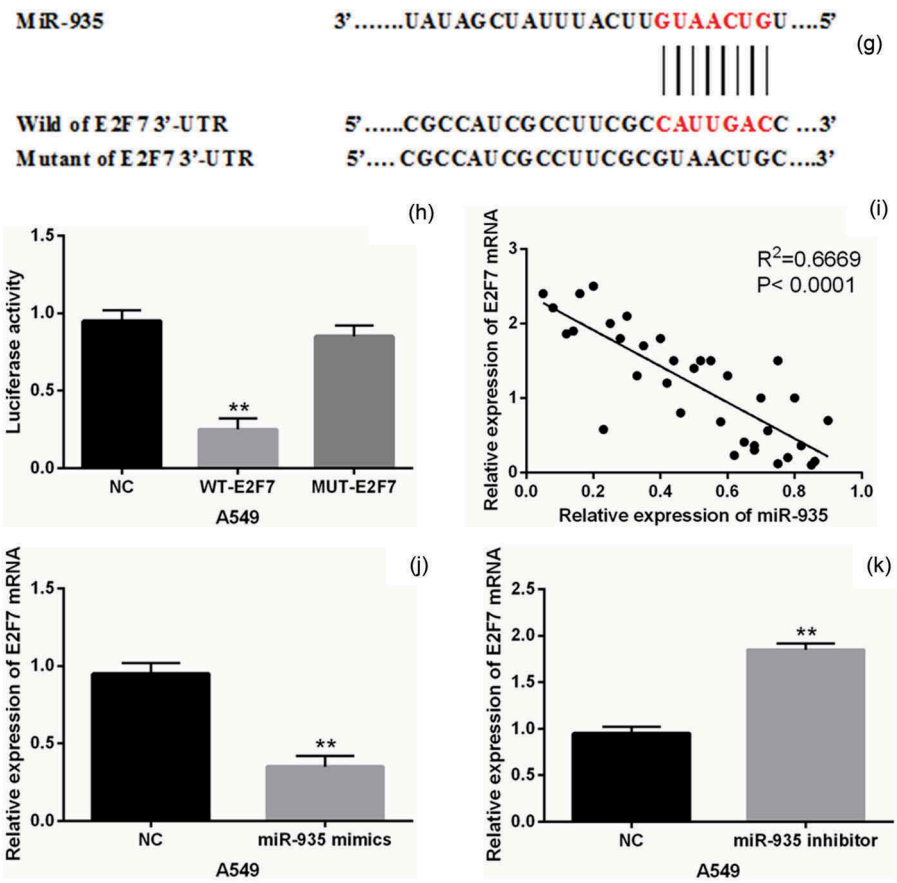

Figure 2. Continued.

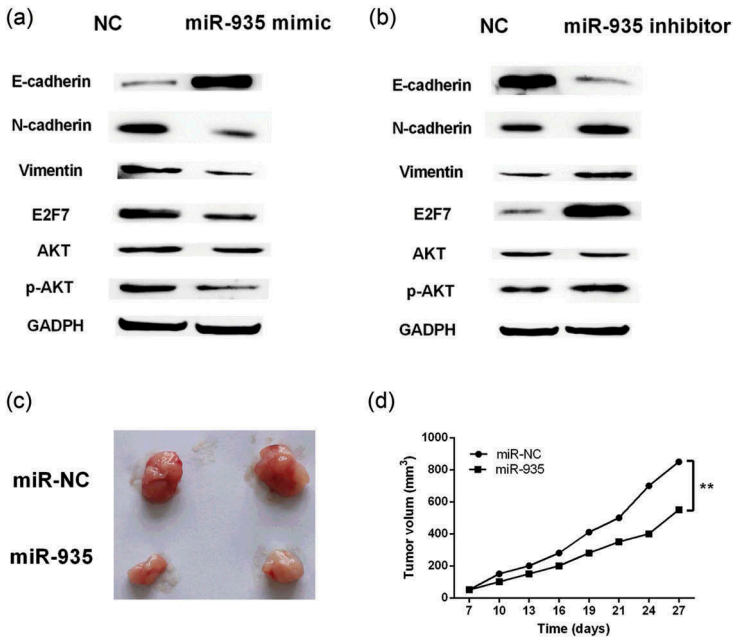

Figure 3. (a, b) Western blot analysis of E-cadherin, N-cadherin, vimentin, AKT and p-AKT in A549 cells contained miR-935 mimics or inhibitor. MiR-935 inhibited the tumour growth *in vivo*. (c) The tumourigenic ability of A549 cells with miR-935 stable transfection plasmid was reduced. (d) The growth rate of tumours with miR-935 stable transfection plasmid was reduced. $^{**}p < 0.01$.

Summary table

What is known about this subject:
- microRNA-935 (miR-935) is implicated in the pathogenesis of certain cancers.

What this paper adds:
- Downregulation of miR-935 in NSCLC tissue predicts poor prognosis.
- E2F7 is a direct target gene of miR-935.
- MiR-935 is likely to suppress the development of NSCLC via targeting E2F7 and inhibiting the AKT pathway.

Disclosure statement

No potential conflict of interest was reported by the authors.

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