Original Article
MicroRNA-25 regulates small cell lung cancer cell development and cell cycle through cyclin E2

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Abstract: Purpose: We intended to examine the underlying mechanism of microRNA-25 (miR-25) in regulating small cell lung cancer (SCLC). Methods: The miR-25 expression was measured by quantitative RT-PCR (qRT-PCR) in 5 SCLC cell lines and 9 human SCLC tissues. In SCLC cell line H510A cells, endogenous miR-25 was downregulated by stable transfection of antisense oligonucleotide of miR-25 (miR-25-as). Then the effects of miR-25 downregulation on SCLC growth, invasion and chemoresistance were assessed by MTT, migration and cisplatin assays, respectively. Furthermore, the effects of miR-25 downregulation on cancer cell cycle arrest, production of cell cycle proteins cyclin E2 and CDK2 were examined by cell cycle assay, western blot and luciferase assays, respectively. Finally, cyclin E2 was over-expressed in H510A cells to investigate its effect on miR-25 mediated SCLC regulation. Results: In both SCLC cells and human SCLC tumor tissues, miR-25 was overexpressed. Down-regulation of miR-25 in H510A cells significantly reduced cancer cell growth, invasive capability and resistance to cisplatin. Also, it induced G1 cell cycle arrest and downregulated cell cycle related proteins cyclin E2 and CDK2. Luciferase assay demonstrated cyclin E2 was directly targeted by miR-25. Overexpression of cyclin E2 in H510A cells reversed the cell cycle arrest and restored invasive capability impaired by miR-25 downregulation. Conclusions: Our study shows miR-25 is overexpressed in SCLC and acting as oncogenic regulator by regulating cyclin E2.

Keywords: SCLC, miR-25, cyclin E2, CDK2

Introduction
Lung cancer is the second most common cancer in the world, accounting for 10%–15% of all new cases of cancers worldwide, and one of the leading cause of cancer-related death in the United States [1]. Small-cell lung cancer (SCLC) is a distinct form of lung cancer with unique clinical and histological characteristics, representing ~15% of all new cases of lung cancer with more than 150,000 cases every year in the world [1]. In contrast to non-small cell lung cancer (NSCLC), SCLC is often associated with lung cancer patients of intensive history of smoking, especially in the developing nations such as China [2, 3]. There is no cure for SCLC and combined chemotherapy is the standard treatment for patients with SCLC [4]. Despite the great emphasis on early diagnosis and best possible care management, the prognosis in SCLC remains very poor. Median survivals for SCLC patients without care, with limited-stage disease, or with extensive-stage are ~4 months, ~20 months (with 2-year survival rate of ~40%), or ~10 months (with 2-year survival rate of ~5%), respectively [5].

MicroRNAs (miRNAs) are families of 17- to 23-nucleotide-long small and non-coding RNAs that exert genetic regulation or posttranslational modulation by directly binding on the 3’-untranslated region (3’-UTR) of target messenger (m) RNAs [6]. It has been shown, both experimentally and clinically, that miRNAs play critical roles in the pathogenesis of human cancers, functioning as both oncogenes and tumor suppressors [7-16]. MiR-25, a putative oncogene has been shown to be overexpressed in a variety of cancers and played functional roles in many malignancy-related processes, such as cancer growth and proliferation, cancer apoptosis, cancer migration and metastasis [17-20]. Interestingly in lung cancers, including non-small cell and small cell lung cancers, miR-25...
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was also found to be overexpressed in carcinoma [21, 22], yet its molecular mechanisms and functional regulations in lung cancer are largely unknown.

Cyclin E2 is a member of cyclin E family, and its major function is making transition from G0/G1 to S phase through the binding of CDK2 and phosphorylation of Rb protein [23, 24]. In breast cancer, overexpression of Cyclin E2 and CDK2 was found to be highly associated with cancer patients with poor survival [25, 26]. In lung cancer, study had showed the expression pattern of cyclin E2 did not have prognostic relevance for survival in patients with NSCLC [27]. However, little is known about the expression as well as the functional mechanism of cyclin E2 in SCLC.

In our study, we found miR-25 was overexpressed in both SCLC cancer cell lines and SCLC tumor tissues. Based on this novel finding, we down-regulated miR-25 in SCLC cell line H510A cells to examine its molecular mechanism in SCLC, including cancer cell proliferation, migration and chemoresistance to cisplatin. We then discovered that miR-25 also regulated SCLC cell cycle by inducing G0/G1 arrest and down-regulating cycle cycle proteins cyclin E2 and CDK2. Through functional experiments including luciferase reporter assay, western blot and recombinant gene overexpression, we further revealed that cyclin E2 was directly involved in the regulation of miR-25 in SCLC.

Materials and methods

Cell lines and culture

Five SCLC cell lines, H146, H209, G446, H510, H889, and fetal lung fibroblast cell line MRC5 were purchased from Institute of Biochemistry and Cell Biology of Chinese Academy of Science in Shanghai, China. The cells were maintained in DMEM (Invitrogen, USA) supplemented with 10% FBS Invitrogen (Invitrogen, USA), 100 IU/ml penicillin, 100 mg/mL streptomycin sulfate and 2 mM glutamine, and in a humidified atmosphere of 5% CO₂ at 37 C.

Patients and clinical samples

From November 2012 to July 2014, 9 pairs of SCLC tumor tissues (T) and matched adjacent non-tumor tissues (ANT) were surgically obtained from patients in the department of General Thoracic Surgery at the Affiliated Hospital of Logistics University of Chinese People's Armed Police Forces in Tianjin, China. Consent forms were signed by all participating patients. All clinical procedures were approved by the Ethic Committee at the Affiliated Hospital of Logistics University of Chinese People's Armed Police Forces. There was no chemotherapy or radiotherapy before surgery. All samples, after surgical extraction, were snap-frozen in liquid nitrogen and stored at -80 C.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by TRizol reagent per manufacturer’s protocol (Invitrogen, USA). Reverse-transcribed complementary DNA was manufactured by a Prime-script RT reagent Kit according to manufacturer’s instruction (TaKaRa, China). RT-PCR was conducted using a SYBR Premix Ex Taq according to manufacturer’s instruction (TaKaRa, China). For miR-25 detection, the forward primer was 5'-GTG-TTGAGAGGCCGAGACTT-3', and the reverse primer was 5'-TCAGACCGACAAGTGCAA-3'. For cyclin E2 detection, the forward primer was 5'-CCTCATGGATGATAAGGACAG-3' and the reverse primer was 5'-CACAATTAGTGGTTTCTTTTCA-3'. Quantification was performed with TaqMan probes and normalized by U6 small nuclear RNA for miR-25, GAPDH for cyclin E2.

MicroRNA transfection assay

The miR-25 anti-sense oligonucleotides inhibitor (miR-25-as), and negative control (miR-Ctl) were produced by Shanghai GenePharma Company (Shanghai, China). For stable transfection, SCLC cell line H510A cells were plated in 6-well plates (5 × 10⁵ cells/well) and transfected with 100 nM miR-25-as or miR-Ctl by Lipofectamine 2000 reagent according to manufacturer’s instruction (Invitrogen, USA).

Cell proliferation assay

SCLC cell line, H510A cells were plated in 96-well plates (5 × 10⁵ cells/well). After transfection with either nM miR-25-as (100 nM) or miR-Ctl (100 nM), cell proliferation was performed by a Cell Titer 96® Aqueous One Solution Assay according to manufacturer’s instruction (Promega, USA). The reagent solutions were added to each well for 3 hours at 37 C. One, two, three and four days after transfection.
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tion, quantification of cancer cell proliferation was conducted by measuring the absorbance at 490 nm with a CytoFluor II multiwell plate reader (PerSeptive Biosystems, USA).

Cell invasion assay

H510A cells were transfected with either miR-25-as or miR-Ctl. The invasion of cells was assessed by counting the number of cells invading Matrigel-coated Transwell chambers through 3 mm pores (Becton Dickinson, USA). Twelve hours after transfection, H510A cells were trypsinized, and 200 mL of cell suspension (2 × 10^5 cells/mL) under each condition were added in triplicate wells. Twenty-four hours after incubation, total cells that migrated into the lower wells were fixed with 70% and quantified by gentian violet assay.

Chemoresistance assay

In order to measure the chemoresistance of SCLC cells to cisplatin, H510A cells were plated in 96-well plates (5 × 10^5 cells/well) and transfected with either miR-25-as (100 nM) or miR-Ctl for 24 hours, followed by 12 hours treatment of cisplatin at various concentrations of cisplatin (1, 2, 5, 10, 20, 50, 100 and 200 mg/ml). Cell viability was assessed by proliferation assay.

Cell cycle assay

H510A cells were plated in 96-well plates (5 × 10^5 cells/well) and transfected with either miR-25-as (100 nM) or miR-Ctl (100 nM). Seventy-two hours after transfection, cells were trypsinized and collected by centrifugation. The cells were then fixed by 70% ethanol for 48 hours at 4 C, rehydrated by ice-cold PBS, and stained by PI/RNase medium containing 1 mg/ml RNase and 50 μg/ml propidium iodide. Cells were then sorted by fluorescence-activated cell sorter scan (FACS) according to manufacturer’s instruction (Becton Dickinson, USA). The percentage of cells in each cell cycle phase was analyzed by an ELITE software (Phoenix Flow Systems, USA).

Western blot assay

H510A cells were plated in 96-well plates (5 × 10^5 cells/well) and transfected with either miR-25-as (100 nM) or miR-Ctl (100 nM). Forty-eight hours after transfection, proteins under each condition were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SS-PAGE) (Invitrogen, USA), and then transferred to polyvinylidene difluoride membranes (Bio-Rad, USA). The membranes were blocked with skim milks and treated with primary antibodies against CDK2 (1:300, Sigma-Aldrich, USA), cyclin E2 (1:300, Sigma-Aldrich, USA) and β-actin (1:1000, Sigma-Aldrich, USA), followed by horseradish peroxidase-conjugated secondary antibodies.

Luciferase reporter assay

A wild type (WT) 3'-UTR fragment of human cyclin E2 mRNA containing the putative miR-25 binding site was amplified by regular PCR and cloned into a pGL3-control vector according to manufacturer’s instruction (Promega, USA). A mutant (MT) cyclin E2 3'-UTR with modified fragments at miR-25 binding site was inserted into pGL3- vector by Site-Directed Mutagenesis Kit (SBS Genetech, China). Both WT and MT sequences were verified by DNA sequencing. Then, H510A cells were transfected with either cyclin E2 WT 3'-UTR plasmid or cyclin E2 MT 3'-UTR plasmid, renilla luciferase pRL-TK vector (Promega, USA), and miR-25-as or miR-Ctl with a Lipofectamine 2000 reagent according to manufacturer’s instruction (Invitrogen, USA). The relative luciferase activities were assessed by a Dual-Luciferase Reporter Assay (Promega, USA) 48 hours after transfection. The signals were normalized to the Renilla luminescence of control vectors under each condition.

Overexpression assay

In order to overexpress cyclin E2 in SCLC cells, recombinant plasmid eukaryotic expression vector pcDNA3.1-CyclinE2 and empty control vector (pcDNA3.1-Ctl) were purchased from Cell Signaling Technology (Cell Signaling Technology, USA). Then, H510A cells were plated in a 6-well plate (5 × 10^5 cells/well) and transfected with either pcDNA3.1-CyclinE2 or pcDNA3.1-Ctl using Lipofectamine 2000 reagent. The transfection efficacy was verified by qRT-PCR 48 hours after transfection.

Statistical analysis

All experiments were conducted in triplicates. The data were shown as the mean ± standard errors. Statistical analyses were made with a windows-based SPSS software (version 11.2).
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Figure 1. MiR-25 is upregulated in SCLC cell lines and human SCLC tissues. A. qRT-PCR was used to assess miR-25 expression levels in MRC5, H146, H209, H446, H510A and H889 cells. All data were normalized to MRC5 (*, P < 0.05). B. qRT-PCR was used to compare the miR-25 expression levels between 9 pairs of tumor tissues (T) and adjacent non-tumor tissues (ANT). All data were normalized to ANT of each case (*, P < 0.05).

Results

MiR-25 expression is upregulated in SCLC cell lines and human SCLC tissues

We measured miR-25 expressions in normal fetal lung fibroblast cell line MRC5, and 5 SCLC cell lines, H146, H209, H446, H510A and H889 by qRT-PCR. We found that, as compared to the expression level of miR-25 in MRC5, the expression levels of miR-25 in all five SCLC cells were significantly upregulated (Figure 1A). We then compared the miR-25 expressions in human SCLC tumor tissues (T) and paired adjacent non-tumor tissues (ANT) in 9 patients (Figure 1B). Similar to the result obtained from SCLC cell lines, miR-25 was significantly upregulated in tumor tissues of all patient samples, as compared to the paired non-tumor tissues (Figure 1B). These results thus suggest a molecular mechanism of miR-25 in SCLC.
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**Downregulation of miR-25 reduces proliferation, invasion and chemoresistance to cisplatin in SCLC cells**

In 5 of the SCLC cell lines, we noticed H510A cells had the highest expression level of miR-25. Thus, we decided to down-regulate the expression of miR-25 in H510A cells to investigate the regulatory mechanism of miR-25. To downregulate miR-25, H510A was transfected with 100 nM miR-25 anti-sense oligonucleotides inhibitor (miR-25-as) for 24 hours. The transfection efficiency was verified by qRT-PCR when compared with the miR-25 expression in H510A cells transfected with 100 nM control miRNA (miR-Ctl) (**Figure 2A**).

We then investigated the functional roles of down-regulating miR-25 in SCLC, including cell proliferation, invasion and chemoresistance.

For that purpose, we transfected SCLC cell line H510A cells with either miR-25 inhibitor (miR-25-as, 100 nM) or non-specific control miRNA (miR-Ctl, 100 nM).

Firstly, we examined whether miR-25 downregulation had any effect on SCLC cell growth through a proliferation assay. The result showed that 72 and 96 hours after transfection, miR-25 downregulation significantly reduced cancer cell proliferation in H510A cells (**: *P < 0.05**). **Figure 2B**.

Secondly, we examined whether miR-25 downregulation had any effect on SCLC cell invasion through a MTT migration assay. The results demonstrated that in H510A cells transfected with miR-25-as, had significantly smaller num-
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number of cancer cells invaded into the lower chamber (Figure 2C, 2D), suggesting that down-regulating miR-25 reduced invasive capability in SCLC.

Thirdly, we examined whether down-regulating miR-25 had any effect on SCLC’s chemoresistance to cisplatin. Twenty-four hours after transfection, H510A cells were treated with various concentrations (1, 2, 5, 10, 20, 50, 100 and 200 mg/ml) of cisplatin for additional 12 hours. Cell viability was checked by the MTT proliferation assay. It showed that, at concentrations between 10 and 100 mg/ml, cisplatin resistance was significantly reduced in H510A cells with miR-25 downregulation (Figure 2E, *: P < 0.05).

Downregulation of miR-25 induced cell cycle arrest through cyclin E2 in SCLC cells

We then investigated whether down-regulating miR-25 had an effect on SCLC cell cycle transition. H510A cells were transfected with either miR-25-as (100 nM) or miR-Ctl (100 nM). Seventy-two hours after transfection, a cell cycle assay was conducted. It showed that, in miR-25 downregulated H510A cells, significantly higher number of cells was in G0/G1 stage as well as significantly lower number of cells in S stage, as compared to H510A cells without miR-25 downregulation (Figure 3A, *: P < 0.05). There was no significant difference in the numbers of cells in G2/M stages between two conditions. Thus, our results demonstrated that miR-25 downregulation induced G0/G1 cell cycle arrest in H510A cells.

Since cell cycle in SCLC cells was modulated by miR-25, we’d like to know whether cell cycle related proteins were also affected by miR-25. Our result of western blotting analysis found that, in miR-25 downregulated H510A cells, two proteins critical to cell cycle, CDK2 and cyclin E2 were significantly down-regulated, as compared to H510A cells without miR-25 downregulation (Figure 3B).

Based on our western blot result, we speculated some of the cell cycle protein might be directly regulated by miR-25 in SCLC cells. We thus conducted a bioinformatic search of the molecular target of miR-25 through online web service TargetScan (http://www.targetscan.org/), and found that cyclin E2 is likely the direct target of miR-25 (Figure 3C). We then inserted the fragments of wild type (WT) cyclin E2 3'-UTR, with putative miR-25 binding site, into pGL3 vector and used a Renilla luciferase reporter assay to examine the possible binding of miR-25 on cyclin E2 in H510A cells. For control experiment, a pGL3 plasmid with mutated (MT) cyclin E2 3'-UTR was also included in the luciferase assay. The result showed that the relative luciferase activity was significantly modified while WT cyclin E2 3'-UTR was co-transfected with miR-25-as (Figure 3D, *: P < 0.05), suggesting that miR-25 was indeed directly acting on cyclin E2 in SCLC cells.

Overexpressing cyclin E2 reversed the effects of miR-25 downregulation in SCLC cells

As we demonstrated that cyclin E2 was directly regulated by miR-25 in SCLC cells, we then investigate whether cyclin E2 had a functional role in regulating miR-25 induced SCLC regulation. To do so, we constructed the cyclin E2 overexpressing vector by inserting cyclin E2 promoter sequence into recombinant plasmid eukaryotic expression vector pcDNA3.1 (pcDNA3.1-CyclinE2). The control vector contains an empty sequence, pcDNA3.1-Ctl. Then, H510A cells were transfected with either pcDNA3.1-CyclinE2 or pcDNA3.1-Ctl for 48 hours. The transfection efficiency and specificity was verified by qRT-PCR (Figure 4A, *: P < 0.05)

We then conducted a dual-transfection experiment in H510A cells. Cells were firstly transfected with miR-25-as for 24 hours, followed by second transfection with either pcDNA3.1-CyclinE2 or pcDNA3.1-Ctl. Three days after transfection, a cell cycle assay demonstrated that cyclin E2 overexpression significantly reversed the G0/G1 cell cycle arrest in H510A cells induced by miR-25 down-regulation (Figure 4B, *: P < 0.05). A matrigel migration assay was also conducted (Figure 4C). It showed that impaired invasion induced by miR-25 downregulation was reversed by cyclin E2 overexpression in H510A cells (Figure 4D, *: P < 0.05). Thus, these results strongly suggest that miR-25 induced regulation was through cyclin E2 in SCLC cells.

Discussion

MicroRNA25 acts as an oncogene in a variety of cancers [17, 19, 20]. In lung cancer, miR-25
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was shown to be overexpressed in patients with NSCLC [21], but little is known of the expression of miR-25 in patients with SCLC. In our study, for the first time, we demonstrated that miR-25 was overexpressed in five SCLC cell lines and SCLC tumor tissues, when compared with normal lung fibroblast cell line MRC5 and adjacent non-tumor tissues, respectively. Thus, along with existing evidence of miR-25 expression in SCLC [28], miR-25 appears to be overexpressed in the majority of lung cancers, despite its SCLC or NSCLC. These results strongly suggest that miR-25 might have functional mechanisms in lung cancers.

Also in the current study, we took further step to assess the molecular mechanism of miR-25 in SCLC. As our results demonstrated miR-25 was overexpressed in SCLC, we down-regulated miR-25 in SCLC cell line H510A cell to see whether miR-25 was acting as an oncogene. Our results supported this hypothesis, showing that miR-25 downregulation reduced H510A cell proliferation, invasion and chemoresistance to cisplatin. Moreover, miR-25 downregulation induced G0/G1 cell cycle arrest and impaired the product of key cell cycle proteins, cyclin E2 and CDK2 in H510A cells. It is worth noting that in another form of lung cancer, NSCLC, though miR-25 was overexpressed in patients’ serum, the correlation between miR-25 and patients’ survival prediction was not established [22]. These evidence suggest that though miR-25 is commonly overexpressed in

Figure 4. Overexpressing cyclin E2 restored cell cycle and invasion impaired by miR-25 down-regulation in H510A cells. A. H510A cells were transfected with recombinant plasmid eukaryotic expression vector pcDNA3.1-CyclinE2 to over-express CDC42, or empty control vector (pcDNA3.1-Ctl). Two days later, cyclin E2 expressions were examined by qRT-PCR (*: $P < 0.05$). B. H510A cells were transfected miR-25-as for 24 hours, followed by another transfection of either pcDNA3.1-CyclinE2 or pcDNA3.1-Ctl. A cell cycle assay was conducted 72 hours after last transfection. (*: $P < 0.05$). C. Twenty-four hours after last transfection, a Matrigel migration assay was conducted to assess the invasive capability of cells. D. Quantification showed significantly more cells invaded into lower chambers while cyclin E2 was overexpressed in H510A cells (*: $P < 0.05$).
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all forms of lung cancers, the exact mechanism of miR-25 might be complex and differ between SCLC and NSCLC.

Various molecular pathways appear to be the targets of miR-25 in various cancer forms. In breast cancer, miR-25 activates TGF-β signaling pathway to induce tumorigenesis [18]. In hepatocellular carcinoma, the cluster miR-106b-25/miR-17-92 activates c-Myc and E2F1 to induce tumor growth and metastasis [19]. Cyclin E2 is a critical tumor regulator in various cancers by affecting the transitions between cell cycle stages [25, 26]. In lung cancer, though cyclin E2 is overexpressed in several SCLC cell lines [29], the exact function of cyclin E2 or its possible association with miRNA regulations was largely unknown. In our study, we demonstrated that cyclin E2 was directly suppressed upon miR-25 downregulation in A510A cells. Furthermore, overexpressing cyclin E2 rescued the impairments on cancer cell growth, cell cycle development and invasive capability induced by miR-25 downregulation. Thus, our results strongly suggest that miR-25 regulates SCLC through a novel molecular pathway in cyclin E2.

Overall, we demonstrated in current study, down-regulating miR-25 inhibited SCLC growth, invasion and chemoresistance to cisplatin, as well as induced G0/G1 cell cycle arrest by directly acting through novel molecular pathway cyclin E2. Targeting miR-25 or its downstreaming molecular targets could be a future treatment option for patients suffered from small cell lung cancer.

Disclosure of conflict of interest

None.

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