MicroRNA-30e reduces cell growth and enhances drug sensitivity to gefitinib in lung carcinoma

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ABSTRACT

MicroRNAs (miRNAs) play critical roles in various biological processes, including malignancy. Here, we demonstrated that miR-30e levels were markedly reduced in human lung carcinoma specimens in comparison with adjacent normal tissues. In addition, miR-30e amounts were starkly lower in the resistant PC9/gefitinib (PC9G) cancer cells compared with PC9 cells. Meanwhile, miR-30e overexpression in PC9G cells resulted in reduced cell proliferation and migration, reversing drug resistance to gefitinib. Conversely, miR-30e silencing in PC9 cells increased proliferation as well as migration, and conferred resistance to gefitinib. Moreover, HOXA1, which was identified as a new miR-30etarget, plays important roles in regulating cell fate, early developmental patterns and organogenesis. Importantly, miR-30e also inhibited PC9G growth in vivo. Taken together, these findings demonstrated that miR-30e should be considered a tumor suppressor miRNA, which could be used in treating human lung cancer.

INTRODUCTION

Lung carcinoma is the top killer among cancers, claiming 1.4 million lives in the world yearly. Non-small-cell lung carcinoma (NSCLC) is found in about 80% of primary lung cancer patients, who are usually diagnosed in advanced stage despite current efforts and improvements aimed at early diagnosis [1]. Although a variety of therapeutic options are available for lung cancer patients, e.g. surgery, chemotherapy and radiotherapy, five-year survival rates remain critically low. In patients with epidermal growth factor receptor (EGFR) activating mutation, treatment with EGFR tyrosine kinase inhibitors (TKIs), e.g. gefitinib, shows high efficacy [2]. However, acquired TKI resistance hampers the use of such molecules. Increasing evidence suggests that miRNAs may significantly affect the development and chemoresistance of lung cancer [3–6].

MiRNAs are endogenous single-stranded non-coding RNAs associated with various types of cancer [7]. They have essential functions in gene regulation, and affect many important pathophysiological processes such as differentiation, development and tumorigenesis [8–10]. MiRNAs mainly bind to the 3′-untranslated region (3′-UTR) of mRNA molecules, suppressing protein synthesis through mRNA degradation or translational repression [11]. Known miR-30e targets include Bmi1, P4HA1, BCR-ABL and UBC9 [12–15]. However, how miR-30eregulates lung cancer tumorigenesis remains unclear.

HOX genes belong to a highly conserved subgroup of the Homeobox superfamily which characteristically
encode a 60-amino acid long DNA-binding motif. HOX genes play critical roles in regulating cell fate as well as early developmental events and organogenesis [16–18]. Alterations in HOX genes are also associated with multiple cancers in humans, e.g. lung, breast, and hematological cancers [19–21]. Here, we demonstrated that miR-30e targeted HOXA1, whose expression was reduced in miR-30e treated cells and conversely enhanced after miR-30e inhibition.

It has been reported that miR-30e may play important roles in cancer by affecting different signaling pathways. The current data revealed that miR-30e amounts were reduced in PC9G cells in comparison with PC9 cells. We further characterized miR-30e and explore its molecular mechanisms in lung cancer. Interestingly, ectopic expression of miR-30e resulted reduced cell proliferation and migration, with induced apoptosis in lung cancer cells by suppressing the key target HOXA1. In addition, miR-30e rendered PC9G cells more sensitive to gefitinib in vitro and in vivo. These findings revealed a novel mechanism for miR-30e, indicating that this miRNA could be further assessed for the development of lung cancer therapeutics.

**RESULTS**

MicroRNA-30e is markedly downregulated in lung carcinoma

To assess the role of miR-30e in lung cancer, miR-30e amounts were assessed in 30 lung cancer tissues with the corresponding adjacent normal tissuespecimens. RT-qPCR showed that miR-30e was significant downregulated in lung cancer tissues (Figure 1A). Then, human lung cancer specimens were divided into 2 groups based on sensitivity of gefitinib; interestingly, lower amounts of miR-30e were obtained in lung cancer patients with gefitinib resistance (Figure 1B). To evaluate the effect of miR-30e on NSCLC patientprognosis after treatment with EGFR-TKI, the Kaplan-Meier method and log-rank test were used to examine normalized miR-30e levels and Disease free survival (DFS). Individuals displaying low miR-30e levels showed reduced DFS as well as overall survival (OS) compared with those displaying elevated miR-30e amounts (Figure 1C, 1D). These findings suggested that loss of miR-30e may be associated with lung cancer disease progression, and should be considered a potential new biomarker for predicting poor prognosis in NSCLC.

PC9G cells shows higher activity of proliferation and migration, and lower apoptosis rates compared with PC9 cells

Gefitinib-based chemotherapy is considered the cornerstone in treating advanced lung cancer. To mimic long-time exposure of patients to gefitinib, an in vitro model was established by transforming human lung cancer PC9 cells via exposure to lower concentrations of gefitinib for 24 weeks (Figure 2A). Interestingly, miR-30e amounts in PC9 cells were elevated compared with values obtained in the resistant PC9G cell line (Figure 2B). PC9G cells had resistance features, including enhanced cell proliferation and migration, alongside lower apoptosis rates (Figure 2C–2E). In addition, we found that PC9G showed increased cell proliferation and migration, with reduced apoptosis compared with PC9 cells.

High miR-30e levels in PC9G cells inhibit cancer aggressiveness and reverses drug resistance to gefitinib

MiR-30e amounts in resistant PC9G cells were lower than in PC9 cells. Interestingly, cell growth was reduced in miR-30e-overexpressing lung cancer cells in comparison with those transfected with miR-NC (Figure 3A and 3B). We next assessed the impact of miR-30e on cell migration. As shown in Figure 3C, miR-30e re-expression starkly reduced the migrationability of lung cancer cells. What’s more, overexpression of miR-30e promoted cell apoptosis (Figure 3D). We further found that miR-30e overexpression reversed drug resistance to gefitinib in PC9G cells (Figure 3E). Thus, our results suggest miR-30e inhibited tumor aggressiveness, and reversed drug resistance to gefitinib.

Repression of miR-30e in PC9 cells significantly promotes cell growth and migration, also conferring resistance to gefitinib

To evaluate miR-30e function in lung cancer carcinogenesis, PC9 cell growth was assessed after transfection with miR-30e-inhibitor. Interestingly, markedly decreased miR-30e levels were observed after silencing of the miRNA, resulting in increased PC9 cell growth compared with the miR-NC-inhibitor group (Figure 4A). Since migration is a very important malignancy feature, the effects of miR-30e on cell migration was evaluated. As shown in Figure 4B, miR-30e-inhibition dramatically induced the normally strong migration capacity of lung cancer cells, promoting cell survival by inducing apoptosis (Figure 4C). Furthermore, inhibition of miR-30e conferred resistance to gefitinib in PC9 cells (Figure 4D). Thus, these results suggest that repression of miR-30e in PC9 cells significantly promoted cell growth and migration, while conferring resistance to gefitinib.

MiR-30e sensitizes HCC827/GR cells to gefitinib

Next, we adopted the human lung cancer cell line HCC827 with its variant HCC827/GR cells which exposure to indicated lower concentration gefitinib for 24 weeks (Figure 5A). Interestingly, miR-30e amounts
in HCC827 cells were increased compared with the values of the resistant HCC827/GR cells (Figure 5B). Meanwhile, HCC827/GR cells had resistance features, including elevated cell proliferation and a reduced apoptosis rate (Figure 5C–5D). Furthermore, high miR-30e amounts reversed drug resistance to gefitinib in HCC827/GR cells (Figure 5E). Meanwhile, inhibition of miR-30e promoted chemoresistance to gefitinib in HCC827 cell lines (Figure 5F). Our results suggested that miR-30e sensitized HCC827/GR cells to gefitinib.

**HOXA1 is a direct target of miR-30e**

To explore the underlying mechanism of miR-30e in lung cancer, the database TargetScan (www.targetscan.org) was searched. We found that miR-30e likely regulates the HOXA1 gene since its 3’-UTR harbored the binding site for the seed region of miR-30e. HOXA1 has a critical function in normal tissue growth and differentiation. Based on the putative binding site of miR-30e in the 3’UTR of the HOXA1 gene, we initially constructed two types of plasmids containing the luciferase reporting gene with wild-type or mutant HOXA1 3’UTR, and co-transfected miR-30e mimics or inhibitor into PC9G or PC9 cells; interestingly, cells co-transfected with miR-30e mimics and wild-type HOXA1 3’UTR showed a significant decrease in luciferase activity, while miR-30e-inhibitor significantly increased the luciferase activity. However, in the mutant group, no detectable change in luciferase activity was observed (Figure 6A–6B), suggesting that miR-30e suppressed the transcription activity of the HOXA1 gene by targeting the putative 3’UTR of HOXA1 mRNA independently. Western blot demonstrated that HOXA1 protein amounts were reduced in miR-30e treated PC9G cells, and increased after miR-30e-inhibition in PC9 cells (Figure 6C). Furthermore, HOXA1 amounts were assessed in human lung cancer samples and adjacent normal tissue specimens, with markedly increased values found in cancer specimens (Figure 6D). Next, the association of HOXA1 content with miR-30e amounts in human lung cancer specimens was assessed by Spearman’s rank correlation. Interestingly, HOXA1 content and miR-30e levels were inversely correlated in human lung cancer specimens (Spearman’s correlation \( r = -0.5382 \)) (Figure 6E). These data demonstrated that miR-30e directly targeted HOXA1 in lung cancer cells.

**Figure 1: MiR-30e expression is markedly reduced in lung cancer.** (A) Relative miR-30e amounts analyzed by qRT-PCR in 30 human lung cancer tissues alongside adjacent non-cancerous specimens, with U6 employed for normalization. (B) MicroRNA-30e amounts were reduced in lung cancer patients resistant to gefitinib. (C, D) Kaplan-Meier curves showing disease-free and overall survival based on miR-30e amounts. High and low miR-30e amounts were defined based on the 50th percentile value. Data represent mean ± SD of three replicates. * and ** indicate significant differences at \( P < 0.05 \) and \( P < 0.01 \), respectively.
MiR-30e enhances the chemosensitivity of gefitinib in vivo

To evaluate the function of miR-30e in cancer aggressiveness in vivo, PC9G/miR-NC and PC9G/miR-30e cells were subcutaneously administered into both posterior flanks of male BALB/c nude mice. The formed tumors were measured every other day; gefitinib was administered by peritoneal injection. Interestingly, MiR-30e decreased tumor volumes and weights compared with the miR-NC group, and miR-30e plus gefitinib resulted in decreased tumor volumes and weights compared with miR-30e (Figure 7A–7C). To explore the molecular mechanisms by which miR-30e affects tumor growth, total protein samples were obtained for Western blot; as expected, HOXA1 protein amounts were reduced in miR-30e expressing tumors (Figure 7D). These findings suggested that miR-30e enhanced lung cancer sensitivity to gefitinib in nude mice.

DISCUSSION

MicroRNAs have significant functions in carcinogenesis, with some correlated with clinical characteristics and outcomes [22]. Meanwhile, lung cancer tumorigenesis involves both genetic and epigenetic alterations, including the induction of oncogenes and/or suppression of tumor suppressors. Altered miRNA expression is commonly found in human carcinomas, e.g. NSCLC [23, 24]. Here, the role of miR-30e in lung carcinoma was evaluated, as well as the underlying molecular mechanisms.

Mounting evidence suggests that miR-30e is a potential tumor suppressor in multiple cancers. As shown above, reduced miR-30e levels were obtained in lung cancer specimens, in comparison with adjacent non-cancerous tissue samples. The expression of miR-30e was reduced in the resistant lung carcinoma PC9G cell line in comparison with PC9 cells. In agreement, miR-30e overexpression resulted in decreased cell growth and migration, while inducing apoptosis in PC9G cells; conversely, miR-30e repression markedly promoted cell growth and migration, and inhibited apoptosis in PC9 cells. This study is the first to reveal miR-30e overexpression in chemosensitivity. These findings could help develop novel therapeutic strategies for lung carcinoma treatment.

HOXA1, a member of the HOXA family which was first identified in Drosophila, has been previously reported to significantly influence the normal growth and differentiation of mammalian tissues [25, 26]. HOXA1 re-expression in human mammary epithelial cells was shown to cause oncogenic transformation...
and tumorigenesis in vivo [27]. HOXA1 mutation results in decreased mammary cell proliferation, anchorage-independent growth, and loss of contact inhibition [28]. In addition, HOXA1 expression levels in squamous cell lung-and cervical cancer tissue samples are significantly elevated compared with adjacent normal tissue specimens [19, 29]. Here, the HOXA1 oncogene was further identified as a new miR-30e target both in vitro and in mice. First, luciferase reporter assay demonstrated that miR-30e directly recognized the 3′-UTR of HOXA1 mRNA. In addition, HOXA1 levels were significantly reduced after stable miR-30e expression. Thirdly, HOXA1 and miR-30e were inversely correlated in clinical specimens. Taken together, these findings indicated that HOXA1 is a new miR-30e target.

MicroRNAs are considered to be involved in cancer chemoresistance; indeed, they are differentially expressed in chemo-sensitive and chemoresistant cells [30–32]. Interestingly, ectopic miR-34a was shown to sensitize colorectal carcinoma cells to 5-fluorouracil [33]; meanwhile, miR-497 reduces tumor cell proliferation and sensitizes to 5-fluorouracil by inactivating KSR1 [34], and targets N-RAS to increase temozolomide-dependent apoptosis in gliomas [35]. As shown above, miR-30e enhances chemosensitivity to gefitinib, both in vitro and in vivo, confirming that miR-30e re-expression may constitute a novel strategy for overcoming chemoresistance to gefitinib in lung cancer. However, the majority of clinical samples did not undergo EGFR mutation assessment because most patients were administered gefitinib after unsuccessful chemotherapy and disease progression. This is a limitation if this study. Therefore, it remains unclear whether the observed response may be due to EGFR mutations. We then retrospectively assessed samples with EGFR mutation, which also showed elevated miR-30e amounts. These findings indicated that elevated miR-30e amounts may overlap to some extent with EGFR mutations, which is a very well-known predictive marker of response to EGFR-TKIs. Further research is required to test this hypothesis.

In summary, the current findings firstly demonstrated that miR-30e played a significant role in suppressing lung cancer cell growth through HOXA1 inhibition. Although we confirmed that miR-30e could inhibit lung cancer by targeting HOXA1, there might be other miR-30e targets, which could also affect tumor growth in lung cancer. Therefore, further studies are warranted for identifying

Figure 3: MicroRNA-30e overexpression in the PC9G cell line reduces cell proliferation and migration, and reverses drug resistance to gefitinib. (A) Real-time PCR quantifying miR-30e amounts in PC9G cells. (B) The CCK8 assay was used to quantitate cell viability after transduction with miR-30e or miR-NC. (C) Transwell migration assays were conducted for respective cells. (D) Apoptosis Assay was carried out for respective cells. (E) Gefitinib sensitivity of the PC9G/miR-NC and PC9G/miR-30e cell lines was tested by CCK-8 assay. Data are mean ± SD of 3 replicate experiments. * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.
Figure 4: Repression of miR-30e in PC9 cells significantly promotes cell growth and migration, and confers resistance to gefitinib. (A) The CCK8 assay of PC9 cells was performed after transduction with miR-30e-inhibitor or miR-NC-inhibitor. (B) Transwell migration assays were carried out for respective cells. (C) Apoptosis assay was performed in respective cells. (D) Gefitinib sensitivity in PC9/miR-NC-inhibitor and PC9/miR-30e-inhibitor cell lines was tested by the CCK-8 assay. Data are mean ± SD of 3 replicate experiments. * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

Figure 5: MicroRNA-30e renders HCC827/GR cells more sensitive to gefitinib. (A) In comparison with the HCC827 cell line, HCC827/GR cells displayed less sensitivity to gefitinib. (B) MicroRNA-30e amounts in HCC827 and resistant HCC827/GR cells. (C) Apoptosis Assay were conducted in HCC827 and HCC827/GR cells. (D) The CCK8 assay of HCC827 and HCC827/GR cells was carried out at various time points. (E) Gefitinib sensitivity in HCC827/GR/miR-NC and HCC827/GR/miR-30e cell lines evaluated by the CCK-8 assay. (F) Gefitinib sensitivity in HCC827/miR-NC-inhibitor and HCC827/miR-30e-inhibitor cell lines assessed by the CCK-8 assay. * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.
additional targets and pathways modulated by miR-30e. Although the mechanisms underpinning lung cancer are currently more understood, treating this malignancy still constitutes a clinical challenge.

MATERIALS AND METHODS

Cell culture and clinical tissue specimens

Human lung cancer PC9, PC9G, HCC827 and HCC827/GR cells were maintained in RPMI 1640; the HEK-293T cell line was cultured in DMEM containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 mg/mL streptomycin. Cell culture was carried out at 37°C in a humid atmosphere with 5% CO2. PC-9 and HCC-827 cellsharbor the activating EGFR mutation del E746-A750 in exon 19.

Lung cancer tissue samples and adjacent non-cancerous tissue specimens were obtained from patients undergoing lung cancer resection, and snap-frozen in liquid nitrogen post-surgery.

Lentiviral packaging of miR-30e and stable cell line establishment

A lentiviral packaging kit was used to stably overexpress miR-30e in lung cancer cells. Lentivirus carrying miR-30e or negative control (miR-NC) was

Figure 6: HOXA1 is a miR-30e target. (A) MicroRNA-30e binding site in the human HOXA1 3′-UTR and a reporter construct depicting the whole HOXA1 3′-UTR fragment as well as the mutant HOXA1 3′-UTR (mutated nucleotides are shown in red). (B) Luciferase assay on PC9G or PC9 cells, co-transfected with mimics or inhibitor and a luciferase reporter comprising full length HOXA1 3′-UTR (WT) or mutated (MT) sequence with 4 changed nucleotides in the miR-30e binding site. Luciferase activity was assessed 24 h after transfection. MicroRNA-30e starkly reduced luciferase activity. Data are mean ± SD (n = 4). (C) The expression of HOXA1 in cells was determined by western blotting analysis. (D) HOXA1 amounts in non-cancerous tissues and human lung cancersamples were assessed by RT-qPCR; fold changes were derived for HOXA1 based on GAPDH amounts. (E) The association of HOXA1 amounts and miR-30e levels was determined by Spearman's correlation. Data are mean ± SD of three replicate experiments. * and ** indicate significant differences at P < 0.05 and P < 0.01, respectively.
packaged according to the manufacturer’s instructions, in HEK-293T cells using polybrene (Sigma-Aldrich); selection was performed by treatment with puromycin (Sigma-Aldrich) for 2 weeks, to yield stable cell lines.

**RNA purification and real-time reverse transcription polymerase chain reaction (qRT-PCR)**

Total RNA was obtained from cultured cells with TRIzol reagent (Invitrogen, USA) as instructed by the manufacturer. Quantitative real-time RT-PCR detecting mature miR-30e was carried out in triplicate with RT Reagent Kit (Vazyme, Nanjing, China) as directed by the manufacturer, withAceQ SYBR Master Mix (Vazyme, Nanjing, China) on a 7900HT system. MicroRNA-30e levels in each group were determined relative to U6 amounts, by the $2^{-\Delta\Delta C_{t}}$ method.

**Cell proliferation assay**

Cell counting Kit-8 (CCK8 kit, Dojindo Laboratories, Japan) assay was used for cell viability assessment. A total of 2,000 cells were plated per well in 96-well plates, and cultured as described above for 48 h after transfection. After incubation for indicated times, CCK-8 reagent was supplemented per well and further incubated for 1–2 h. Absorbance was read at 450 nm.

**Migration assay**

The effects of miR-30e on cell migration were investigated in 24-well Matrigel invasion chambers (BD Biosciences, UK) as instructed by the manufacturer. Transfected cells ($5 \times 10^{4}$) were plated in upper wells in serum-free RPMI-1640, with RPMI-1640 containing 10% FBS in lower chambers. After 16–20 h, non-invasive cells (top wells) were removed; invasive cells (bottom wells) were submitted to staining with 0.1% crystal violet after fixation (paraformaldehyde). Photomicrographs were captured in 3 randomly selected high power fields. After air drying, the membranes were treated with 33% acetic acid (300 µL/well) at room temperature for 15 minutes, and the resulting solutions transferred into 96-well plates. Absorbance at a wavelength of 570 nm was recorded.

**Western blotting**

Cells were treated as described above for 48 h, and lysed in RIPA buffer containing protease inhibitorson ice for 30 min. Total protein amounts were assessed by the BCA assay (Beyotime, China). Equal amounts

![Figure 7](image.png)

**Figure 7: MiR-30e enhances chemosensitivity to gefitinib in a mouse model.** (A–C) MicroRNA-30e affects PC9G cell growth in nude mice. BALB/c nude mice were subcutaneously administered $5 \times 10^{6}$ cells transduced with lentiviruses carrying miR-NC or miR-30e. The tumors were measured at different time points; gefitinib treatment was carried out intraperitoneally. The tumors were extracted and weighed at 24 days. MicroRNA-30e treatment resulted in decreased tumor volumes and weights compared with the miR-NC group; meanwhile, miR-30e plus gefitinib further inhibited tumor growth compared with the miR-30e group. Bar = 1 mm. (D) HOXA1 amounts were assessed in tumor tissue specimens by immunoblotting. Data are mean ± SD. * and # indicate significant differences at $P < 0.01$ and $P < 0.05$, respectively.
of protein were then separated by 10% SDS-PAGE. Subsequently, protein bands were electrically transferred onto nitrocellulose membranes (Whatman, Germany), which were incubated with anti-HOXA1 (Proteintech Technology, USA) and anti-GAPDH (Bioworld Technology, USA) antibodies at 4°C overnight.

Luciferase reporter assay

TargetScan was employed to predict miR-30e binding sites. A fragment of the 3′-UTR of HOXA1 with the putative miR-30e binding site was cloned by PCR. To generate a construct harboring mutated miR-30e binding site, four nucleotides corresponding to the 5′-seeding region of this site were substituted in the wild type fragment. The complementary fragment in the 3′-UTR of HOXA1 (UGUUUAU) was mutated to UCAUAUC. PCR products were cut with SacI and HindIII, cloned inserted into pMIR-REPORTER, and validated by DNA sequencing. Constructs were then co-transfected with miR-30e or miR-NC into HEK-293 cells in 24-well plates for 24 h, followed by Luciferase assays with Dual Luciferase Reporter Assay System (Promega, WI, USA).

Apoptosis assay

Apoptosis was assessed flow-cytometrically, afterstaining with AnnexinV and propidium iodide (BD Pharmingen) according to the manufacturer’s instructions. Analysis was carried out on FACSCanto II (BD Biosciences) with the FlowJo software.

Xenograft studies

BALB/cA-nu (nu/nu) nude mice (male, 6-weeks old), purchased from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China), were housed in a specific pathogen-free (SPF) vivarium. Aliquots of cells (5 × 10⁶) in 150 mL FBS-free RPMI 1640 was subcutaneously administered into both posterior flanks of the animals. Tumors were measured with Vernier calipers every 2 days, with volumes derived as followed: Volume = 0.5 × Length × Width². Ten days after implantation, gefitinib (5 μM) was intraperitoneal injected in the indicated mice. The animals were euthanized 24 days after implantation, extracting the tumors.

Statistical analysis

Experiments were carried out in triplicate, with data assessed using GraphPad Prism 5 (La Jolla, CA, USA). The association of miR-30e levels with HOXA1 amounts in human lung cancer was assessed by Spearman’s rank test. Group comparison was carried out by t-test. Statistical significance level was set at P < 0.05.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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