MiR-130a Overcomes Gefitinib Resistance by Targeting Met in Non-Small Cell Lung Cancer Cell Lines

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Abstract
Non-small cell lung cancer (NSCLC) is the most common type of lung cancer and the most common cause of lung cancer death. Currently, the epidermal growth factor receptor inhibitor gefitinib is used for its treatment; however, drug resistance is a major obstacle. Expression of Met has been associated with both primary and acquired resistance to gefitinib, but the mechanisms regulating its expression are not fully understood. Recently, miRNAs such as miR-130a have been shown to play a role in gefitinib resistance, but importance in NSCLC and relationships with Met have not been fully explored. Here we show that miR-130a is over-expressed in gefitinib-sensitive NSCLC cell lines, but is low in gefitinib-resistant NSCLC cell lines. Moreover, miR-130a expression was negatively correlated with that of Met. Further analysis revealed that over-expression of miR-130a increased cell apoptosis and inhibited proliferation of NSCLC cells treated with gefitinib, whereas lowering the expression of miR-130a decreased cell apoptosis and promoted cell proliferation after treatment with gefitinib in both gefitinib-sensitive and -resistant NSCLC cell lines, suggesting that miR-130a overcomes gefitinib resistance. We also demonstrated that miR-130a binds to the 3'-UTR of Met and significantly suppresses its expression. Finally, our results showed that over-expressing Met could “rescue” the functions of miR-130a regarding cell apoptosis and proliferation after cells are treated with gefitinib. These findings indicate that the miR-130a/Met axis plays an important role in gefitinib resistance in NSCLC. Thus, the miR-130a/Met axis may be an effective therapeutic target in gefitinib-resistant lung cancer patients.

Keywords: miR-130a - gefitinib resistance - Met - NSCLC

Introduction
Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers and is the most common cause of lung cancer death (Molina et al., 2008). Elucidation of the molecular abnormalities in NSCLC has led to the development of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib (Oxnard et al., 2010; Sekido 2010). These are both used to treat patients with locally advanced or metastatic NSCLC. Thus, EGFR-TKIs have been suggested for use as a first line therapy for patients with NSCLC harboring EGFR mutations. However, despite this and other advances in lung cancer therapy, patient survival is still quite poor. This is in part due to the development of drug resistance (Dimitroulis et al., 2005; Burris 2009).

Met protein is the receptor tyrosine kinase for hepatocyte growth factors. Its expression and phosphorylation have been associated with both primary and acquired resistance to EGFR-TKI therapies in NSCLC patients (Engelman et al., 2007; Suda et al., 2010). Thus, Met has been implicated as a potentially effective therapeutic target to reverse resistance to this important class of drugs in NSCLC.

MicroRNAs (miRNAs) are a class of small non-coding RNAs, and contain approximately 22 nucleotides. miRNAs bind to partially complementary sequences in the 3’-untranslated regions (UTRs) of specific target mRNAs, resulting in either mRNA degradation or translation inhibition (Sandberg et al., 2008; Matoulkova et al., 2012). Growing evidence suggests that miRNAs play an important role in various biological processes, including cell proliferation, development and differentiation (Farazi et al., 2011; Kong et al., 2012; Ramachandran et al., 2012). Indeed, Garofalo et al. (2012) found that some miRNAs, such as miR-221, miR-222, and miR-30, are associated with gefitinib resistance in NSCLC cell lines by targeting the proteins which contribute to drug resistance. Some researchers have also shown that miR-130a contributes to multidrug resistance in human hepatocellular carcinoma (HCC) and ovarian cancer (Xu et al., 2012; Yang et al., 2012). However, to date there have been no reports concerning the role of miR-130a in gefitinib resistance in NSCLC.

In this study, we demonstrate that miR-130a is overexpressed in gefitinib-sensitive NSCLC cell lines, but not gefitinib-resistant NSCLC cell lines. Over-expressing
miR-130a was found to increase the sensitivity of NSCLC cells to gefitinib-induced apoptosis, while lowering its expression led to gefitinib resistance. Moreover, we also determined that miR-130a mediates gefitinib resistance by directly targeting Met.

Materials and Methods

Cell lines, cell culture and reagents

Human adenocarcinoma lung cancer cell lines A549, H1975 Pc-9, and Pc-9 gefitinib resistant (Pc-9 GR) cells were bought from the American Type Culture Collection. They were cultured in RPMI1640 with 10% fetal bovine serum in a 5% CO₂ incubator at 37°C with saturated humidity. For transfection, cell lines were cultured to 60% confluence in plates containing serum-free medium without antibiotics. They were then transfected with 100 nmol of miR-130a mimics or inhibitor for 48 h. All transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. GAPDH antibody was purchased from Santa Cruz, city, state, USA, and the Met antibody was purchased from Abcam, city, state, USA.

RNA extraction and real-time polymerase chain reaction (PCR)

RNA was extracted with TRIzol solution (Invitrogen) and the integrity of RNA was assessed with an Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA, USA). Briefly, cells were collected and washed twice with cold phosphate-buffered saline (PBS), and 1 ml of TRIzol was added for 15 min at room temperature (RT) to resuspend the cell pellets. Then, 0.2 ml of chloroform was added and the mixture was centrifuged at 12 000 × g for 15 min. Total RNA was precipitated with isopropanol, then washed with 75% ethanol. Finally, total RNA was dissolved in double distilled water without RNase. Reverse transcription was performed using the RT-PCR kit (Fermentas, USA), and real-time PCR was performed using Applied Biosystems 7300 Real-Time PCR system (USA).

Plasmid construction

The Met over-expressing plasmid (pcDNA3.1-Met plasmid) was constructed in our laboratory. Briefly, full-length Met was isolated from a human fetal liver cDNA library and subcloned into the pcDNA3.1 plasmid. The PCR primer sequences of Met were as follows: forward primer: GGGGTACCAGAGCCCGCCGGCTGTTT, reverse primer: CCCTCGAGCATGATGTCCAGGAAG. PCR products were digested and cloned into KpnI and XhoI site of pcDNA3.1 vectors. PcDNA3.1-Met plasmid was sequenced by Invitrogen (China) and sequencing result was indentified to that in NCBI gene bank.

Western blot

Cells were collected and washed twice with ice-cold PBS and then lysed in 1% NP40 for 30 min at 4°C. Equal amounts of protein lysate were separated on 10% or 12% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Bio-Rad). The membranes were blocked for 1h at RT with 5% nonfat dry milk diluted in 1×TBST (10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% tween 20) and then incubated with specific antibodies overnight at 4°C to detect each protein. After three washes with 1×TBST for 15 min each time, the membranes were incubated for 2 h at RT with secondary antibodies conjugated to HRP in 5% milk-TBST buffer. The membranes were then washed thrice in 1×TBST with buffer changes at 10 min intervals. Blots were visualized with enhanced chemiluminescence (Pierce, city, state, USA) and then detected by a gel imaging system (Alpha Innotech, city, state, USA).

Cell viability assay

Cells were seeded in 96-well culture dishes at a density of 4000/well. The following day, cells were transfected with miR-130a mimics (miR-130) or miRNA mimics negative control (miR-NC), miR-130 inhibitor (Inh-130) or miRNA inhibitor negative control (Inh-NC). Different doses of gefitinib (0–20 μM) were added to cells 24 h later. After a further 72 h, 10 μl CCK8 was added to each well for 1 h. The absorbance was then measured at 450 nm. Each condition was tested in triplicate and the average values were obtained.

Apoptosis assay (Annexin V/PI assay)

Annexin V/PI assay was performed using the Annexin V/PI assay kit (Beyotime, city, China.) according to the manufacturer’s instructions. Briefly, NSCLC cells of about 40–60% confluence were seeded in 12-well plates. Then, cells were transfected with miR-130a mimics or miR-NC and Inh-130a or Inh-NC using Lipofectamine 2000. After 24 h, different doses of gefitinib (0–20 μM) were added into each well. Forty-eight hours later, cells were collected and washed twice with ice-cold PBS and then stained with 5 μl Annexin V and 5 μl PI in a total volume of 100 μl PBS. Cells were kept in the dark for 15 min and then the fluorescent signal was measured. All experiments were performed three times.

Luciferase activity assay

Luciferase activity assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Briefly, HEK293A cells were seeded in 24-well plates at approximately 60–80% confluence. For Met 3’-UTR luciferase reporter assay, 100 ng wild type or mutant luciferase reporter constructs (termed WT or MUT) were co-transfected with 100 nM miR-130a or 100 nM miR-NC using Lipofectamine 2000. The Luciferase activity assay was performed 24 h after transfection using the Dual-Luciferase Assay System. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity. All experiments were performed three times.

Statistical analysis

The data are presented as mean ± standard deviation of the mean (SD). P-values were calculated using an unpaired Student t test. A P-value < 0.05 was considered significant. All statistical analyses were performed using SPSS 18.0 software.
Results

MiR-130a is overexpressed in gefitinib-sensitive NSCLC cell lines and Met is overexpressed in gefitinib-resistant NSCLC cell lines

We first analyzed the effect of gefitinib on the growth of NSCLC cells lines. H1975, A549, Pc9 gefitinib-sensitive (Pc9) and Pc9 gefitinib-resistant (Pc9 GR) cells were treated with different doses of gefitinib (0 µM, 0.01 µM, 0.1 µM, 1 µM, 5 µM, 10 µM, and 20 µM) and cell growth was then detected by the CCK8 method. Consistent with previous studies (Li et al. 2011), the results showed that H1975 and Pc9 cell lines were sensitive to gefitinib whereas A549 and Pc9 GR were relatively resistant (Figure 1A). We next detected the expression of miR-130a and Met in the four NSCLC cell lines by qRT-PCR or western blot. Compared with the gefitinib-sensitive cell lines (H1975 and Pc9), miR-130a expression was significantly lower in the gefitinib-resistant cell lines, A549 and Pc9 GR (Figure 1B). In contrast, Met mRNA expression was notably greater in gefitinib-resistant cell lines than in gefitinib-sensitive cell lines (Figure 1C). Our results also showed that the protein expression of miR-130a was inversely correlated with that of Met.

MiR-130a overcomes gefitinib resistance in NSCLC cell lines

Previous studies have shown that the amplification of Met plays a key role in gefitinib resistance in NSCLC (Engelman et al., 2007). This prompted us to explore the possible biological significance of miR-130a in gefitinib resistance in NSCLC cell lines. As an initial step, we investigated whether miR-130a affected gefitinib-induced apoptosis in the NSCLC cell line, Pc9 GR. Thus, cells were transfected with negative control (miR-NC) or miR-130a mimics (miR-130a) and then treated with gefitinib, and the apoptosis rate was then detected by Annexin V/PI assay. Our results showed that at a dose of 1 µM gefitinib, the apoptosis rate of the miR-130a group was significantly greater than that of the miR-NC group (Figure 2A). Conversely, when the miR-130a inhibitor (Inh-130a) was transfected into Pc9 cells, the gefitinib-induced apoptosis was lower than that of the Inh-NC group (Figure 2B).

We next analyzed the effect of miR-130a on the growth of gefitinib-resistant cells after gefitinib treatment using the CCK8 assay. We found that transient transfection of Pc9 GR and A549 cells with miR-130a, significantly promoted gefitinib-mediated growth inhibition to different degrees (Figure 2C). To provide further evidence that miR-130a supports NSCLC cell growth inhibition in response to gefitinib treatment, we studied the effect of an inhibitor of miR-130a in Pc9 and H1975 cells. Proliferation of cells transfected with Inh-130a was increased in a dose-dependent fashion compared with that of cells transfected with Inh-NC after gefitinib treatment (Figure 2D). Thus, together these findings suggest that miR-130a is involved in mediating the response to gefitinib in NSCLC cell lines. Met is a potential target of miR-130a in NSCLC

miRNA exerts its function by binding to the 3’-UTR of target genes through partial sequence homology. By using a bioinformatic approach, we found that Met is a potential target of miR-130a (http://www.targetscan.org/). To test whether Met could be a biological target of miR-130a, we transfected A549 cells with miR-130a and the miR-NC, as well as the miR-130a inhibitor. Forty-eight hours after transfection, we examined Met protein levels in cells...
We found that Met protein levels were suppressed by miR-130a mimics in A549 cells (Figure 3A). In contrast, transfection of A549 cells with Inh-130a resulted in significantly greater Met protein levels than that of Inh-NC transfected cells (Figure 3B). However, the mRNA level of Met was not significantly changed whether cells were transfected with miR-130a or Inh-130a (Figure 3C), suggesting that miR-130a specifically regulates Met at the protein level.

To further determine whether Met is a direct target of miR-130a, we copied the 3'–UTR of Met into the pGL3 plasmid (Met 3' UTR). We then mutated the binding sites of miR-130a in the Met 3' UTR (Met 3' UTR mut). Luciferase reporter assays were then performed to evaluate the effect of mi-R130a, as well as that of its inhibitor, on Met transcriptional activity in A549 cells. It was found that the mimics of miR-130a suppressed the relative luciferase activity of Met 3' UTR to approximately 35%. However, when the miRNA targeting sequence was mutated in the reporter plasmids, miR-130a mimics did not influence the relative luciferase activity (Figure 3D). Additionally, the inhibitor of miR-130a increased the relative luciferase activity of Met 3' UTR, and this effect was abrogated when the miR-130a binding in Met was mutated (Figure 3E).

MiR-130a reverses gefitinib resistance through down-regulation of Met in NSCLC cells

We postulated that miR-130a reverses gefitinib resistance through down-regulation of Met in NSCLC.

To test the above hypothesis, we developed a ‘rescue’ experiment by co-transfecting the Pc9 GR cells with mimics of miR-130a and a pcDNA3.1 vector carrying Met sequences. First, we transfected Pc9 cells with Met alone and found that it led to gefitinib resistance as demonstrated by increased proliferation (Figure 4A–B). These results are consistent with previous reports (Engelman et al., 2007). In contrast, Western blot demonstrated that co-transfection of Pc9 cells with miR-130a mimics and Met restored miR-130a-induced loss of Met protein expression (Figure 4C). Moreover, retrieval of Met protein expression in Pc9 GR cells by miR-130a mimics overcame gefitinib-resistance in Pc9 GR cells (Figure 4D–E).

Discussion

NSCLC accounts for 85% of all lung cancer and is the most common cause of cancer death. One of the most important factors that affects survival rate is tumor recurrence. This occurs mostly because of drug resistance. Gefitinib, an EGFR inhibitor, is one of the most effective drugs used for the treatment of NSCLC patients (Paez et al., 2004; Sequist et al., 2008; Gazdar 2009). Current
research has indicated that secondary mutations of EGFR and Met amplification are the two most important factors underlying gefitinib resistance in NSCLC (Pao et al., 2005; Kosaka et al., 2006; Engelman et al., 2007). Recently, it has been reported that miRNAs such as miR-221, miR-222, and miR-30, which are regulated by EGFR or Met, contribute to gefitinib resistance by targeting the pro-apoptotic protein BIM in NSCLC cells (Garofalo et al., 2012). In this study, we found that miR-130a was up-regulated in gefitinib-sensitive NSCLC cell lines but was low in gefitinib-resistant NSCLC cell lines. Moreover, our results also showed that miR-130a contributes to gefitinib resistance in NSCLC cell lines. Importantly, our results demonstrated that miR-130a overcomes gefitinib resistance by directly regulating Met expression in NSCLC.

MiR-130a plays an important role in multiple kinds of tumors. For examples, miR-130a is downregulated in prostate carcinoma and jointly supresses two major oncogenic pathway (Boll et al., 2013), miR-130a increases drug resistance by regulating RUNX3 and Wnt signaling in caspstatin-treated HCC cells (Xu et al., 2012), and upregualtion of miR-130a is associated with MDR1/P-glycoprotein-mediated drug resistance in ovarian cancer cells (Yang et al., 2013). Moreover, Mujahid et al. found that miR-130a regulates lung airflow and vascular development in the fetal lungs (Mujahid et al., 2013), and Chen et al found miR-130a regulates angiogenesis by down-regulating GAX and HOXA5 (Chen et al., 2008; Boll et al., 2013). In a previous study, Acunzo et al. (2012) found that miR-130a could target Met and induce TNF-related apoptosis-inducing ligand (TRAIL) sensitivity in NSCLC by down-regulating miR-221 and miR-222. However, to date, there have been no reports concerning the relationship between miR-130a and gefitinib resistance in NSCLC. Thus, for the first time, we report that miR-130a can increase cell apoptosis and inhibit cell proliferation of gefitinib-treated NSCLC cells, whereas down-regulation of miR-130a by its specific inhibitor leads to gefitinib resistance in NSCLC cell lines.

A role for Met in gefitinib resistance was first described in 2007 by Engelman et al. (2007) who reported that Met amplification leads to gefitinib resistance in lung cancer by activating the ERBB3 signaling pathway. Soon afterwards, more researchers reported that Met amplification and its phosphorylation are associated with both primary and acquired resistance to EGFR TKI therapies in NSCLC patients (Guo et al., 2008; Suda et al., 2010; Belalcazar et al., 2012). Together, these reports implicate Met as a potentially effective therapeutic target to reverse resistance to this important class of drugs in NSCLC. Moreover, the Met receptor promotes a complex biological program designated “invasion growth” that results from stimulation of cell motility, invasion and protection from apoptosis (Zhang et al., 2012; Luo et al., 2013). To better understand the up-stream pathways involved in NSCLC gefitinib resistance, we investigated the mRNA, miR-130a, which can regulate Met expression. In this study, we first reported that miR-130a contributed to gefitinib resistance by regulating Met at the protein level, but not the mRNA level. Furthermore, we found that miR-130a suppressed Met protein levels by directly targeting its 3’-UTR. Moreover, we also demonstrated that miR-130 overcomes gefitinib resistance in a Met-dependent manner by conducting a rescue experiment.

In conclusion, our results show that miR-130a is up-regulated in gefitinib-sensitive NSCLC cells, whereas it is down-regulated in gefitinib-resistant NSCLC cells. Moreover, we demonstrate that miR-130a contributes to gefitinib resistance in NSCLC by targeting the Met 3’-UTR and down-regulating Met protein levels. Furthermore, we found that miR-130a-mediated gefitinib-resistance in NSCLC cell lines can be rescued by Met. Our results indicate that miR-130a/Met axis plays an important role in gefitinib resistance in NSCLC and that this axis may be an effective therapeutic target in gefitinib-resistant patients.

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