MicroRNA-30b inhibits non-small cell lung cancer cell growth by targeting the epidermal growth factor receptor

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Lung cancer remains the leading cause of cancer-related death in the world, and its morbidity and mortality have risen significantly in recent years [1]. Non-small cell lung cancers (NSCLCs) account for approximately 85% of lung cancer cases, and most of them are advanced when diagnosed [2]. Surgery is an efficient protocol for NSCLC, however, only approximately 30% patients have the opportunity to undergo curative resection. Other treatment options include chemotherapy and radiation therapy alone or in combination. Regretfully, the overall 5-year survival rate with these therapies is only 15% [3]. Molecular targeted drugs have been the main treatment for advanced NSCLC, which has the advantages of high specificity, obvious curative effect and small side reaction [4]. Treatment with EGFR tyrosine kinase inhibitors (EGFR-TKIs) in patients with lung adenocarcinoma yields a good curative effect. However, the frequent appearance of drug resistance has limited its further development [5]. Therefore, a new molecular marker that is helpful for the diagnosis and prognosis of NSCLC is urgently needed.

MicroRNAs (miRNAs) are small, non-coding RNAs that specifically recognize 3′-untranslated regions (3′-UTR) of targeted mRNA and bind to it to either repress translation or degrade these mRNAs [6]. miRNAs also participate in complex biological processes, such as cell proliferation, differentiation and apoptosis [7], and they can act as oncogenes or tumor suppressor genes in different cancer types [8]. miR-30b is up-regulated in melanoma [9], medulloblastoma [10], malignant mesothelioma [11], head and neck cancer [12] and oral squamous cell cancers [13]. Recently, it was also found that miR-30b functions as a tumor suppressor in colorectal cancer [14], gastric cancer [15] and lung cancer [16]. Moreover, the overexpression of miR-30 also suppresses EMT phenotypes and inhibits cell migration and invasion in prostate cancer [17]. The above findings have prompted us to look into the relationship between miR-30b and EGFR in NSCLC, particularly because EGFR activation not only accelerates tumor progression but also causes acquired resistance to TKIs.

To better clarify the function of miR-30b in NSCLC and its association with the efficacy of EGFR-TKIs, we first examined the expression of miR-30b in 47 paired tissue samples from NSCLC patients. Then we investigated the ability of miR-30b to regulate EGFR in several NSCLC cell lines, and whether miR-30b was associated with proliferation, migration, invasion and apoptosis. We also explored the relationship between miR-30b and the sensitivity to EGFR-TKI in NSCLC cells. In our study, we first identified that EGFR was a direct target of miR-30b, and that miR-30b may act as a therapeutic target in NSCLC.
**Materials and methods**

**Tissue specimens.** All paired tumor and adjacent normal (≥3 cm away from the tumor) tissues were obtained from 47 NSCLC patients in Taizhou Hospital, the affiliated hospital of Wenzhou Medical University (Taizhou, China) between 2010 and 2011. All the samples were freshly frozen in liquid nitrogen and stored at −80°C until further use. Informed consent was obtained from all patients, and we recorded the related clinicopathological information. No patients received chemotherapy or radiotherapy prior to surgery.

**Cell culture.** A293 cells, normal human bronchial epithelial cells (BEAS-2B), and human NSCLC cell lines A549, H1975 and HCC-827 were used in this study. H1975 and HCC-827 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. H1975 has the L858R/T790M EGFR allele, and HCC827 harbors an intrame EGFR E746–A750 deletion. A293, A549, H1975 and HCC-827 cells were cultured in RPMI-1640 medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) with 100 units/mL penicillin and 100 μg/mL streptomycin. BEAS-2B cells were cultured in LHC basal medium (Gibco) containing 10% FBS. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

**RNA isolation and qRT-PCR.** Total RNA was isolated from NSCLC tissues, adjacent normal tissues and cell lines with TRIzol (Sigma, CA, USA). miR-30b and U6 cDNA was synthesized with gene-specific primers by using a TaqMan® Universal Master Mix II and TaqMan® MicroRNA Assays (Applied Biosystems, CA, USA). For the luciferase reporter assay, A293 cells were plated on a 12-well plate and co-transfected with 50nM miR-30b mimics or NC and 200 ng luciferase reporter plasmid using Lipofectamine™3000 (Invitrogen). Cells were collected 48 hours after transfection and measured with dual-luciferase assay kit (Promega, WI, USA) according to the manufacturer’s instructions. The experiment was repeated three times.

**Western blotting.** Total protein was extracted from transfected cell lines, and the protein concentration was measured by the BCA Protein Assay Kit (Promega, WI, USA). The membranes were blocked with 5% non-fat milk or BSA and incubated with anti-EGFR, anti-AKT, anti-ERK, anti-BCL-2 (Abcam, CA, USA), anti-pEGFR, anti-pAKT, anti-pERK (CST, CA, USA) or anti-GADPH (Abcam) antibodies. Following extensive washing with TBST, membranes were incubated with secondary antibodies (Huabio, Hangzhou, China). Immunoreactive protein bands were detected using an ImageQuant LAS500 system (GE Healthcare, CA, USA).

**MTS assay.** For cell proliferation assay, 5000 cells of each cell line were seeded onto 96-well plates and transfected with miR-30b mimics or NCs at a final concentration of 50 nM. To quantitate cell viability with the Cell Proliferation Assay.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>N(%)</th>
<th>Relative expression of miR-30b</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td>0.205</td>
</tr>
<tr>
<td>Male</td>
<td>25(53.2%)</td>
<td>0.369±0.177</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>22(46.8%)</td>
<td>0.432±0.155</td>
<td></td>
</tr>
<tr>
<td>Age(years)</td>
<td></td>
<td></td>
<td>0.321</td>
</tr>
<tr>
<td>&lt;65</td>
<td>23(48.9%)</td>
<td>0.424±0.149</td>
<td></td>
</tr>
<tr>
<td>≥65</td>
<td>24(51.1%)</td>
<td>0.375±0.185</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td>0.546</td>
</tr>
<tr>
<td>No</td>
<td>15(31.9%)</td>
<td>0.420±0.122</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>32(68.1%)</td>
<td>0.388±0.189</td>
<td></td>
</tr>
<tr>
<td>Tumor size(cm)</td>
<td></td>
<td></td>
<td>0.073</td>
</tr>
<tr>
<td>&lt;3</td>
<td>17(36.2%)</td>
<td>0.455±0.183</td>
<td></td>
</tr>
<tr>
<td>≥3</td>
<td>30(63.8%)</td>
<td>0.364±0.152</td>
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</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td>0.586</td>
</tr>
<tr>
<td>AC</td>
<td>32(68.1%)</td>
<td>0.406±0.158</td>
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<tr>
<td>SCC</td>
<td>15(31.9%)</td>
<td>0.371±0.216</td>
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<tr>
<td>Lymphatic metastasis</td>
<td></td>
<td></td>
<td>0.011*</td>
</tr>
<tr>
<td>No</td>
<td>23(48.9%)</td>
<td>0.459±0.179</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>24(51.1%)</td>
<td>0.335±0.133</td>
<td></td>
</tr>
<tr>
<td>TNM stages</td>
<td></td>
<td></td>
<td>0.004**</td>
</tr>
<tr>
<td>I</td>
<td>21(44.7%)</td>
<td>0.475±0.184</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>16(34.0%)</td>
<td>0.380±0.137</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>10(21.3%)</td>
<td>0.269±0.071</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td>0.013*</td>
</tr>
<tr>
<td>Well &amp; moderate</td>
<td>35(74.5%)</td>
<td>0.434±0.174</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>12(25.5%)</td>
<td>0.298±0.101</td>
<td></td>
</tr>
</tbody>
</table>

AC adenocarcinoma, SCC squamous cell carcinoma; * indicated statistical significance (p<0.05), **indicated p<0.01.
Kit (Promega), 10 μl was added to each well after 24, 48 and 72 hours respectively, and then the plates were incubated at 37°C for 2 hours. The absorbance was measured at 490 nm. The experiment was repeated three times.

For the drug sensitivity assay, different concentrations of gefitinib were added into each well after transfection and the cells were incubated at 37°C for 48 hours. Then 10 μl of solution was added, and the remaining parameters were the same as in the cell proliferation assay.

**Transwell assay.** The cell invasion assay was performed in a 24-well plate with 8-μm pore size chamber inserts (Corning, CA, USA). The transfected cells, which were serum-starved for 24 hours, were resuspended in 200 μl of serum-free medium in the upper chambers, which were lined with a membrane coated with 60 μl Matrigel (BD Biosciences, CA, USA), and 500 μl medium containing 10% FBS was added to the bottom chamber. The cells were incubated at 37°C in a 5% CO₂ humidified incubator for 24 hours, and the cells on the upper surface of the membrane were wiped with cotton wool. Cell monolayers on the underside of the membrane were stained with Giemsa. Three visual fields of each chamber were randomly counted under a microscope with 10 × 40 magnification. Each test was performed in triplicate.

For the cell migration assay, with the similar principles and approaches, we used trans-well filters (Corning) without Matrigel on the upper surface of a polycarbonate membrane (6.5 mm diameter, 8 μm pore size).

**Flow-cytometry analysis.** Cells were seeded in six-well plates (2 × 10⁵/well). Flow cytometry was used to analyze apoptosis. After transfection for 24 hours the cells were harvested, and the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 & PI (Invitrogen) was used according to the manufacturer’s instructions. Stained cells were then analyzed using flow cytometry. Experiments were conducted at least three times, each with three replicates.

**Statistical analysis.** All analyses were performed with SPSS 20.0 software. All experiments above were repeated three times. Data are presented as means ± standard deviation (SD). Overall survival was estimated with the Kaplan–Meier method, and the resulting curves were compared using the log-rank test. Significance between groups was analyzed using Student’s t-test and one-way analysis of variance (ANOVA). p<0.05 was considered significant.

**Results**

Down-regulation of miR-30b in NSCLC tissues. We first evaluated the expression of miR-30b in 47 NSCLC and paired adjacent normal tissues by real-time PCR. The results demonstrated that the miR-30b expression levels in NSCLC...
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Figure 2. EGFR was identified as a target of miR-30b. A Bioinformatic analysis predicted the putative binding sequence between miR-30b and wild-type (WT) EGFR mRNA. B Dual-luciferase reporter assay analyses of A293 cells co-transfected with miR-30b and the reporters showed that miR-30b directly recognizes the wild-type 3’-UTR of EGFR (p<0.05). C–E Western blot analysis of EGFR and the protein products of its downstream genes expression in transfect- ed A549, H1975 and HCC-827 cells. EGFR, p-EGFR, AKT, p-AKT, ERK, p-ERK and BCL-2 were dramatically down-regulated in miR-30b over-expressing cells (p<0.05).

Figure 3. miR-30b inhibits NSCLC cell proliferation. A miR-30b expression was assessed by real-time PCR in four cell lines. miR-30b was significantly increased when cells were transfected with mimics, compared to BEAS-2B cells, NC groups and untreated groups of A549, H1975 and HCC-827 cells (p<0.01). B–D Cell growth analyzed by the MTS assay. The proliferation of the miR-30b treated H1975 and HCC-827 groups significantly decreased at 72 hours (p<0.01), while the A549 group showed a significant decrease at both 48 hours (p<0.05) and 72 hours (p<0.01).
tissues were significantly lower than those in paired adjacent normal tissues (p<0.05; Figure 1A). Notably, further analysis revealed that the expression level of miR-30b in NSCLC tissues was associated with TNM stage, differentiation, and lymph node metastases (p<0.05; Table 1; Figure 1B–D). The mean relative expression level (0.399±0.168) of miR-30b in all 47 NSCLC samples was chosen as the cut-off point for separating tumors with low expression (<mean, n=24) and high expression (≥mean, n=23) of miR-30b. Kaplan-Meier survival analysis and log-rank tests revealed that patients with high miR-30b expression had longer survival times, whereas those with low miR-30b expression had shorter survival times (Figure 1E, log-rank, p=0.004).

**miR-30b targets EGFR by binding the EGFR 3’-UTR.**
Two publicly available bioinformatic algorithms, TargetScan and miRANDA were used to analyze target genes of miR-30b, and the results showed that EGFR was a theoretical target gene of miR-30b (Figure 2A). To determine whether miR-30b directly recognizes the EGFR 3’-UTR, we performed a dual-luciferase reporter assay using luciferase reporter vectors containing either the wild-type or the mutant 3’UTR of EGFR. After co-transfection with miR-30b mimics and the reporter plasmid, we found a significant decrease with the wild-type 3’-UTR of EGFR (p<0.05). However, there is a non-significant change with the mutant EGFR (Figure 2B). Western blot analysis also showed that the protein levels of EGFR were dramatically down-regulated in miR-30b over-expressing cells (p<0.05; Figure 2C–E).

**miR-30b inhibits NSCLC cells proliferation.** To investigate the effect of miR-30b on NSCLC cell proliferation, miR-30b mimics were transfected into A549, H1975 and HCC-827 cells. qRT-PCR revealed that miR-30b was significantly increased compared to the level in BEAS-2B cells, NC groups and untreated groups in A549, H1975 and HCC-827 cells (p<0.01; Figure 3A). The proliferation of NSCLC cells was detected by MTS assay. The cellular proliferation of miR-30b treated groups gradually declined compared with that of the NC groups and untreated groups for A549, H1975 and HCC-827 cells, and H1975 and HCC-827 groups showed significant difference at 72 hours (p<0.01), while A549 group showed significant difference at 48 hours (p<0.05) and especially 72 hours (p<0.01; Figure 3B–D). Thus, the results demonstrate that miR-30b could inhibit NSCLC cell proliferation.

**miR-30b inhibits migration and invasion of NSCLC cells.** To better understand the function of miR-30b in human NSCLC cell metastasis, we performed subsequent transwell assays with and without Matrigel. In the migration assays, miR-30b groups significantly inhibited cell migration compared with that observed in the NC groups and untreated groups for A549, H1975 and HCC-827 cells (p<0.01; Figure 4A–B). The invasion assays revealed a prominent reduction of invasive cells in the miR-30b groups (p<0.01; Figure 5A–B), while cells in the NC groups and untreated groups were almost unaffected. Taken together, miR-30b inhibits the migration and invasion of NSCLC cells.

**miR-30b induces the early apoptosis of NSCLC cells.** To explore whether miR-30b could affect NSCLC cell apoptosis, flow cytometric analysis was used to detect it. After treatment with miR-30b mimic or NC, we found that the miR-30b groups underwent significantly more early apoptosis than was observed in the NC groups and untreated groups for A549, H1975 and HCC-827 cells (p<0.01; Figure 6A–B). Western blot analysis also showed that the protein levels of BCL-2 were dramatically down-regulated in miR-30b over-expressing cells (p<0.05; Figure 2C–E). Therefore, miR-30b induces NSCLC cell early apoptosis.

**miR-30b enhances the sensitivity to gefitinib in NSCLC cells.** To investigate whether miR-30b expression could affect gefitinib sensitivity in NSCLC cell lines, different concentrations of gefitinib were added after transfection. We found that the 50% inhibitory concentration (IC50) values of gefitinib after transfection with miR-30b mimics were 36.25 μM, 3.57 μM and 15.07 μM for A549, H1975 and HCC-827 cells, respectively. A549, H1975 and HCC-827
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respectively. The IC50 values of gefitinib after transfection with NC were 56.62 μM, 17.03 μM and 34.33 μM for A549, H1975 and HCC-827 cells, respectively. The IC50 values of gefitinib were 57.68 μM, 17.21 μM, and 36.06 μM for A549, H1975 and HCC-827 cells, respectively. Therefore, miR-30b enhanced the sensitivity to gefitinib in NSCLC cells (p<0.01; Figure 7A–D). However, we also found that A549 cells were not more sensitive to gefitinib than the H1975 and HCC-827 cells after transfection with miR-30b mimics.

Discussion

The poor survival rate of advanced NSCLC with traditional treatment emphasizes the urgent need to find new therapeutic strategies. Recently, attentions have focused on the role of miRNAs in tumorigenesis. Several publications have reported that miR-21, miR-200c, and so on are prognostic factors in patients with NSCLC [18–21]. miR-30b is one of the members of miR-30 family, and accumulating evidence indicates that miR-30b is a suppressor gene in NSCLC. However, the molecular mechanism remains unclear. In our study, we found that the miR-30b expression level was decreased in NSCLC tissues and cells, and was associated with TNM stage, differentiation, and lymph node metastases in NSCLC. miR-30b could also inhibit the migration and invasion of NSCLC cells. These results were consistent with obvious findings [22]. Moreover, we found that patients with high miR-30b expression had longer survival times than those with low expression, and it could also induce the apoptosis of NSCLC cells. To our knowledge, this is the first time it has been shown that miR-30b inhibits proliferation, migration and invasion, and induces early apoptosis of NSCLC cells by targeting EGFR and repressing EGFR expression. Furthermore, miR-30b can enhance the EGFR-TKI sensitivity in NSCLC cells. These results suggest that miR-30b functions as a tumor suppressor and may contribute to metastasis in NSCLC.

EGFR, the cell-surface receptor for members of the epidermal growth factor family of extracellular protein ligands [23], which is expressed in many tumors, is regarded as a biomarker of aggressive disease and shorter
Certain studies have demonstrated that EGFR might be the target gene in several miRNAs. Denoyelle et al. [25] found that miR-491-5p-induced apoptosis in ovarian carcinoma depends on the direct inhibition of both BCL-X and EGFR leading to BIM activation. Zou et al. [26] indicated that miR-107 is a novel promoter of tumor progression that targets the CPEB3/EGFR axis in human hepatocellular carcinoma. Wang et al. [27] suggested that miR-143 inhibits EGFR-signaling-dependent osteosarcoma invasion. Similarly, EGFR might be a target of miR-30b, as suggested by several bioinformatic algorithms. Therefore, we performed luciferase assays and western blot analyses to reveal that miR-30b suppresses EGFR expression by targeting its 3’-UTR. The tyrosine phosphorylation of EGFR leads to activation of many signaling pathways through the recruitment of diverse proteins, such as PI3K-Akt and MAPK signaling pathways, which promote DNA synthesis and cell proliferation, invasion, differentiation, and survival [29–31]. In the present study, we used three NSCLC cell lines to explore the association between miR-30b and the EGFR signaling pathway. Two EGFR mutant-type cell lines (H1975, HCC-827) and one EGFR wild-type cell (A549) were transfected with miR-30b mimics and NC, and the AKT, ERK and BCL-2 pathways were all inhibited following the decrease of EGFR in the miR-30b treated groups for all three cell lines. Regrettably, we did not find any visible differences between EGFR mutant-type cells and EGFR wild-type cells in these pathways. Altogether, above results suggest that miR-30b could regulate the EGFR signaling pathways.

In conclusion, we found that miR-30b was down-regulated in NSCLC tumor tissues and was associated with TNM stage, differentiation, and lymph node metastases, and patients with high miR-30b expression levels had longer survival times. Moreover, miR-30b inhibits proliferation, migration and invasion, induces apoptosis and enhances the EGFR-TKIs sensitivity of NSCLC cells by targeting EGFR and repressing EGFR signaling pathways. Therefore, all these results suggest that miR-30b directly regulates EGFR and it may become a potential therapeutic target to help decrease or even overcome the resistance of EGFR-TKIs in NSCLC patients. Nevertheless, we did not explore what happens when miR-30b is inhibited, and we may analyze these results in vivo.

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References


