Original Article
miR-223 inhibits tumor development of non-small cell lung cancer and sensitizes cancer cells to gefitinib via targeting E2F1

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Abstract: MicroRNAs (miRNAs) are a class of short and non-coding RNA molecules that are capable of inhibiting translation of mRNAs of target genes. Previous studies have revealed that miRNAs are involved in the tumorigenesis and development of lung cancer. The Rnase-resistance of circulating miRNAs made it valuable non-invasive biomarkers and had attracted extensive research interests. The aim of the present study was to investigate the role of miR-223 in non-small cell lung cancer (NSCLC). The miRNA profiling showed a significantly differential expression of miR-205 and miR-223 between NSCLC tissue and normal adjacent tissues. Higher serum miR-233 level is corresponding to better survival, while miR-205 did not show difference regarding correlation of survival rate with its expression level. Using TargetScan predicting server, E2F1 was selected as a candidate target of miR-223, which was validated via luciferase assay. Further, overexpression of E2F1 counteracted the inhibitory effect of miR-223 on cell proliferation, and alleviates the proapoptosis by miR-223. Considering the deregulation of E2F1 had been reported in chemoresistant cells, we investigated the role of miR-223 in gefitinib challenged A549 cells. The tumor volume growth of mice injected with cells carrying lentivirus mediated miR-223 showed significantly decrease than control when both group treated with gefitinib on 15th day, which is consistent with survival analysis of those nude mice. These evidences point to the prospect of utilizing miR-223 in the prognosis of NSCLC patients under chemotherapy.

Keywords: Non-small cell lung cancer, miR-223, E2F1, gefitinib, chemoresistance

Introduction

Lung cancer is one of the most lethal carcinomas among world population, which contributes to 27% of all cancer death [1]. Among various subtypes, non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases and was considered as a refractory subtype, which is probably due to accumulation of mutations in RAS and p53 pathways [2]. Although substantial improvements have been made in diagnosis of lung cancer, the diagnosis rate is still undesired. Particularly, 25% of the diagnosed cases have already developed regional metastasis and 55% of them have distant metastasis, which made the therapy considerably intractable [3]. Indeed, with surgical resection at early stage, the 5-year survival rates is now approaching 70% [4]. However, there is still a demand of better therapies and monitoring strategies for late-stage and aggressive disease that do not depend on particular mutations.

Previous studies revealed distinct protein-coding mutations as well as aberrantly expressed miRNAs profiles. miRNAs are 19-25 nucleotide-long endogenous noncoding RNAs which serve as critical components in regulating gene expression [5]. They bind to the target gene sequence with perfect or imperfect complementarity, thereby regulating the degradation or deterring the transcription of targets. Among those abnormally expressed miRNAs, let-7 and miR-34 are the most frequently reported miRNAs and the reduction of them in NSCLC is particularly associated with oncogenic phenotype of NSCLC, since they target key oncogenes implicated in multiple pathways that are contributory to tumorigenesis and maintenance of
Oncologic properties, such as RAS, MET and MYC [6-8]. Recently, the Rnase-resistant circulating microRNAs (miRNAs) have garnered extensive attention, and were frequently reported as a promising component for early detection and prediction of outcomes of cancers [9]. According to recent estimation, about 50% of annotated human miRNAs are detected in tumor tissues, including lung cancer [10, 11]. Increasing evidence showed that subsets of miRNAs hold promise as diagnostic and prognostic biomarkers in malignancies [12]. Albeit some miRNAs have been recognized, considering the heterogeneity of NSCLC, novel discovery of potential biomarker is still needed.

In this study, we firstly performed miRNA profiling on tumor tissue and their adjacent normal tissues based on a pre-designed list of candidates, and identified miR-205 and miR-223 as research interest due to their significant fold changes between tumor and normal tissues. We then examined this finding in a cohort consisting of 126 patients and 100 normal controls, investigating the miR-223 and miR-205 content in their serum. Consequently, we found miR-223 has striking fold change between patients and normal control, while miR-205 showed much compromised absolute fold change. Furthermore, the individuals with high miR-223 expression presented higher survival rate than those with low miR-223 expression, and this was not observed in miR-205. Lentivirus-mediated overexpression of miR-223 respectively decreased the cell growth and increased the apoptosis, whereas silencing of miR-223 showed the opposite effect. Targetscan predicted E2F1 as a target of miR-223, which was validated by luciferase assay, as well as diminished E2F1 by overexpressing miR-223 and vice versa. As E2F1 has been highly implicated in drug resistance, we further investigated the effect of miR-223 on sensitivity of nude mice transplanted with miR-223 mimics and miR-223 antagonir overexpressing cells to gefitinib. Tumor size was significantly reduced in mice transplanted with miR-223 mimics overexpressing cells, and increased by xenografting cell overexpressing miR-223 antagonist. In addition, significantly elevated survival rate of miR-223 mimics overexpressing xenograft than control further support the hypothesis that miR-223 can be a promising prognostic biomarker.

**Material and methods**

**Patients and samples**

For miRNA expression level assay, three tumor-matched samples were obtained from NSCLC patients who received surgery in Hua’ian People’s First Hospital. For serum miRNA level experiments, 126 patients who were diagnosed with NSCLC at Hua’ian People’s First Hospital during from August, 2011 to September, 2014 were recruited. All studied patients were elder than 18 years of age, and had a pathological diagnosis of NSCLC as per histological or cytological criteria via biopsy or surgical resection. Those patients do not have a history of lung cancer, other cancers, or synchronous multiple cancers, never chemotherapy or radiotherapy before the blood were sampled. Tumor-node-metastasis (TNM) staging was performed as per relevant specification of American Joint Commission. Health control group recruited from the individuals taking physical examination at Healthy Physical Examination Center of Hua’ian People’s First Hospital. The checked items include chemistry profile, baseline electrolytes, blood cell counts, CEA and AFP, C-reactive protein, type-B ultrasound at abdomen and pelvis, and a chest X-ray. Individuals showed abnormality in any one of those items or have findings suggesting pulmonary pathology or constitutional symptoms were excluded. Written informed consents were obtained from patients and healthy individuals, followed by face-to-face interviews regarding demographic origins and locations, as well as exposure to smoking. The ever smokers were defined as those smoked one cigarette per day for over one year, and current smokers were those kept smoking.

**RNA isolation and serum miRNA qRT-PCR assay**

Venous blood samples were collected and centrifuged at 800 g for 12 min at room temperature to separate serum. Another round of centrifugation at 10,000 g at room temperature for 15 min was performed to completely remove cell debris. Total RNA was extracted using Trizol Reagent (Invitrogen), which was then reversed transcribed to cDNA using AMV reverse transcriptase (Takara). Real-time PCR was conducted with Taqman miRNA probes. All experiments
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were performed triplicate. For miRNA level detection of tumor-matched samples, the relative amounts of miRNA were normalized to internal miRNA controls RNU6B. For serum miRNA level detection, Ct values were determined using the fixed threshold settings, which were then corrected according to standard amplification curve of series synthetic miRNA oligonucleotides of known concentrations. Since RNU6B can be degraded in serum, the expression levels of miRNAs were directly normalized to total RNA.

Cell culture and miRNAs transfection

The human non-small cell lung cancer cell line A549 was procured from Shanghai Institute of Cell Biology. The A549 cells were cultured in RPMI 1640 (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen) at 37°C, 5% CO2 in humified incubator. After growing to 70%-80% confluence, A549 cells were transfected with miR-223 mimics, antagonir or scramble sequence as control (Thermo Fisher Scientific, US) for 24 h at roomtemperature. Transfection was mediated by lentiviral vector GV259 (Shanghai GeneChem Co., Ltd.). The A549 cells were seeded at 5×104 with 2×106 lentivirus-transducing units in the presence of 10 µg/ml polybrene in a 24-well plate. miR-223 mimics and miR-223 antagonir were synthesized by GenePharma (Shanghai, China). For drug-resistance assay, medium containing 0.1 µmol/L gefitinib was used to replace the original medium after cell adhesion.

Cell viability assay

A549 cells were seeded in 96-well plates at a density of 2×105 cells/cm2 with 100 µl cell culture medium. Cell viability was assessed using Cell Counting Kit 8 (CCK-8, Dojindo Laboratories) at different time points: 0 h, 24 h, 48 h and 72 h by a microplate reader by spectrophotometry at 450 nm.

Western blot assay

Cells were lysed in NP-40 buffer containing 150 mM NaCl, 1.0% NP-40, 50 mM Tris-HCl and protease inhibitors (Roche) and 1 mM phenylmethyl sulfonylfluoride for 30 minutes on ice. The supernatant was subject to protein denaturation with buffer containing 2% sodium dodecylsulfate (SDS; Sigma), was subsequently analyzed using SDS-PAGE and monoclonal antibody against E2F1, PCNA and cleaved caspase-3. The resultant gel was then washed and covered by appropriate horseradish peroxidase-conjugated Ig secondary antibodies. Enhanced chemiluminescence (GE Healthcare) was used to detect the staining.
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**Table 1.** Characteristics of patients and their serum content of miR-223 and miR-205

<table>
<thead>
<tr>
<th>Variable</th>
<th>NSCLC (n=126)</th>
<th>Control (n=100)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>55</td>
<td>45</td>
<td>0.893</td>
</tr>
<tr>
<td>&gt;60</td>
<td>71</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>67</td>
<td>51</td>
<td>0.9472</td>
</tr>
<tr>
<td>Ever</td>
<td>28</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>31</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Histological types</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>47</td>
<td></td>
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</tr>
<tr>
<td>Large cell carcinoma</td>
<td>15</td>
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<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>35</td>
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<td></td>
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<tr>
<td>II</td>
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<td>III</td>
<td>52</td>
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<td></td>
</tr>
<tr>
<td>IV</td>
<td>28</td>
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<td></td>
</tr>
<tr>
<td>Other diseases</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>None</td>
<td>117</td>
<td>98</td>
<td>0.2586</td>
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<tr>
<td>Cardiac dysfunction</td>
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</tr>
<tr>
<td>Active infection</td>
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<td>2</td>
<td></td>
</tr>
<tr>
<td>Neurologic disorders</td>
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<td></td>
<td></td>
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<tr>
<td>miR-205</td>
<td>25.77±6.78</td>
<td>15.50±5.47</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-223</td>
<td>20.05±7.54</td>
<td>40.82±9.18</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Apoptosis by flow cytometry**

Annexin V and propidium iodide (PI) flow cytometry was used to measure apoptosis in fibroblasts. Fibroblasts were plated in 6-well plates at a density of 2×10^5 cell/cm^2. After 48 hrs transfection with miR-223 mimics and antagonirs, 100 μL cells were suspended in 1× Annexin-binding buffer and then incubated with 5 μL fluorescein isothiocyanate (FITC) annexin V and 5 μL PI (Invitrogen, US) for 15 min at room temperature in darkness. After adding 400 μL to each replicate, the viability and apoptosis were analyzed by flow cytometry under 530 nm and >575 nm, respectively (BD Bioscience).

**Luciferase assay**

E2F1 3'UTR and its mutated counterpart sequence plasmids were constructed. Cells were seeded in a 24-well plate at cell density of 1×10^5 cells/well. After 24 hours of culture, cells were co-transfected with firefly luciferase reporter plasmids respectively containing wild-type or mutant E2F1 3'UTR pRL-TK vector expressing Renilla luciferase, and miR-223 or miRNA negative control via Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured after 36 hours by Dual Luciferase Reporter Assay (Promega, US). Each transfection was performed twice in triplicate.

**In vivo experiments**

Around 4-6 weeks old male nude mice were purchased from Shanghai Experimental Animal Center of the Chinese Academy of Sciences, which were housed under specific pathogen-free conditions at room temperature and less than 40% humidity. Excess food and water were provided. For tumor growth assay, 15 mice were randomly allocated to 3 groups: Control, Gefitinib treated, and miR-223+ Gefitinib. A total of 2×10^6 control A549 cells and A549 cells overexpressing miR-223 were subcutaneously injected into the lower quadrant of the mice. When the tumor reached 80-90 mm^3, gefitinib was intragastrically administered to each mouse for 16 days on daily basis. The tumor size was evaluated every 3 days for 4 weeks using calipers. The tumor volume was calculated as V=(length × width^2)/2. Further, to analyze the survival of different groups, 15 mice were randomly allocated into control, Gefitinib treated, and miR-223+ Gefitinib group. Gefitinib administration was conducted when the tumor size reached 70-90 mm^3. After the mice suffered from limitations in activities and significant weight loss, they were sacrificed by cervical dislocation. All the animal raise and
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experiment were approved by the Institutional Animal Care and Use Committee of Huai’An People’s First Hospital.

Statistical analysis

Each biological replicate has three technical replicates to calculate the average value of each parameter. Data are reported as mean ± SD. Kaplan-Meier analysis was applied to analyze survival data. Student’s t-test was used to test the significance of differences, and P<0.05 was considered significant.

Results

miR-223 and miR-205 were differentially expressed between NSCLC and normal adjacent tissues

We quantified 60 miRNAs in NSCLC and corresponding normal adjacent tissues to identify the miRNAs that were differentially expressed between them. Absolute fold change and p-value calculated based on t-test were used as measurement of differential expression. As shown in Figure 1A, miR-205 and miR-223 had
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Further, the serum content of miR-205 and miR-223 were examined in 126 NSCLC patients and 100 normal people. The clinical characteristics of those patients were described in Table 1. The average age and smoking status were not significantly different between two groups. miR-205 was upregulated in the serum of NSCLC patients, while miR-223 was downregulated, both with fold change less than that found in between miRNA profiling of cancer and normal adjacent tissues.

Overexpression of miR-223 suppresses proliferation and promotes apoptosis of A549 cells

In previous section, we demonstrated that miR-223, instead of miR-205, is associated with survival rate of NSCLC patients. However, more direct evidence implicating its suppressive role in progression of NSCLC are still needed. Thus, we used the human NSCLC cell line A549 transfected with miR-223 mimics and antagonirs to investigate whether miR-223 inhibit the progression of NSCLC cells. Cell proliferation and apoptosis were two events critical for cancer progression. Overexpression of miR-223 by transfecting miR-223 mimics resulted in reduced cell proliferation and higher apoptosis percentage in A549 cells, and down-regulation of miR-223 by transfecting miR-223 antagonirs produced the opposite effects (Figure 2A-C).

E2F1 is a target of miR-223

Since miRNAs function by mediating partial or entire degradation of mRNAs of target genes, determination of target genes are critical for elucidating the role of miR-223 in NSCLC. To address this, we adopted Targetscan, a tool based on searching for the presence of 8 mer, 7 mer, and 6 mer sites that match the seed region of each miRNA, to predict target genes.
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of miR-223. Among the 412 transcripts, we chose E2F1 since it is a transcription factor and has been implicated in cancer development. E2F1-3’-UTR and E2F1-3’-UTR with mutation sequences plasmids were constructed (Figure 3A), then cotransfected with miR-223 mimic or scrambled control plasmids into A549 cells. After culturing for 24 hours, the fluorescent intensity was detected. The miR-223+E2F1-3’-UTR WT group showed significantly higher luciferase activity than the miR-miR-223+E2F1-3’-UTR MUT group (P=0.00046) (Figure 3B). Western blot results showed that the expression of E2F1 in A549 cells transfected with miR-223 mimics was obviously lower than those transfected with miR-223 antagonirs and blank control (Figure 3C). mRNA expression level of E2F1 also present similar pattern (Figure 3D).

Based on the effect of miR-223 on cell proliferation and apoptosis, we selected biomarkers as molecular indication for evaluating the above biological processes: PCNA for cell proliferation and cleaved caspase-3 for apoptosis. Consistent with what was observed in A549 cells transfected with miR-223 mimics and antagonirs, overexpression of miR-223 increased PCNA level and reduced the content of cleaved caspase-3 (Figure 3C).

Overexpression of E2F1 counteracts the effect of miR-223 on proliferation and apoptosis

To further examine that the effect of miR-223 on cell proliferation and apoptosis was due to reduction of E2F1, overexpression of E2F1 in A549 cells was performed. The plasmids respectively carried miR-223 mimics and E2F1 were cotransfected into A549 cells. E2F1 overexpression was found to alleviate the inhibition of miR-223 mimics on cell proliferation, and the apoptosis rate was significantly reduced (Figure 4A, 4B), which is consistent with protein level of biomarkers detected by western blot (Figure 4C).

miR-223 enhances the sensitivity of NSCLC to gefitinib in vivo

Since we have demonstrated that NCLSC patients with high miR-223 expression have significantly higher survival rate than the patients with low miR-223 expression, another question arise as we attempted to hypothesize that miR-223 could serve as a prognosis biomarker. Drug resistance is an important factor affecting the prognosis of cancer patients; therefore, we sought to investigate whether miR-223 could sensitize NSCLC to anti-cancer drugs. To address this question, an in vivo model was established by subcutaneous injection of miR-223-GV259 infected cells into mouse skins under left lower quadrants. All
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mice were divided into 3 groups: control, control + gefitinib and miR-223 mimic + gefitinib. Tumor sizes were measured every 3 days. Except control group, control + gefitinib and miR-223 mimic + gefitinib group were intragastrically administered with gefitinib from 15th day for 2 weeks. miR-223 mimic + gefitinib group were injected with miR-223-GV259 and treated with gefitinib on 15th day for 2 weeks. Gefitinib exerts anti-cancer effect on both groups, and miR-223 mimic + gefitinib has significantly lower tumor volumes than control + gefitinib (*P<0.05), gefitinib was administered on 15th day for 2 weeks for Control + gefitinib and miR-223 mimic + gefitinib groups.

Discussion

The important roles of miRNAs have been indicated in multiple lines of studies investigating tumor initiation and progression [13-17]. The deregulation of miRNAs is highly implicated in cancer biology. miR-223 was reported in hematopoietic system for the first time [18], which initiated subsequent characterization of its biological functions. Previous studies revealed that miR-223 was down-regulated in human embryonic stem cells, nasopharyngeal cancer cells, and HeLa cells [19-21]. In addition, the inhibitory role of miR-223 in cell proliferation, colony formation and migration were further investigated and determined [21, 22]. In lung cancer, the anti-oncogenic role of miR-223 has also been characterized. In the present study, we observed a significantly higher fold change of miR-223 against control in the serum blood of NSCLC patients, compared with other miRNAs in the profiling.

E2F transcription factors are situated downstream of growth factor signaling cascades, and regulate genes required for cell cycle progression by acting as transcriptional activators or suppressor. Therefore, the role of E2Fs in tumor initiation and development is ambivalent: both oncogenic and suppressive depending on the cellular context. E2F1 was recognized as a potent regulator of apoptosis when DNA damage occurs in various type of human cancer [23]. It was reported that tumor cells, especially from advanced lesions may survive diminished cell death pathways when E2F1 was deregulated in the absence of RB [24] a cell-death effector that acts with cytochrome c and caspase-9 to mediate p53-dependent apoptosis. Loss of Apaf-1 expression is accompanied by allelic loss in metastatic melanomas, but can be recovered in melanoma cell lines by treatment with the methylation inhibitor 5-aza-2'-deoxycytidine (5aza2dC. Previous studies revealed that E2F1 is a tumor suppressor in colon carcinomas [25]. However, it is suggested that overexpression of E2F1 may result in gene amplification, which confers proliferative advantage to the tumor cells in lung and liver colon cancer metastasis [26] n=9. Abnormal expression of E2F1 or aberrant amplificaticn...
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tion of E2F1 gene has been implicated in a variety of human cancers. Multiple lines of studies suggested that elevated E2F1 expression was predominantly associated with advanced tumor stages and metastasis status, as well as poor prognosis. In bladder cancer, Lee and colleagues established a model taking account of expression of E2F1 and the associated genes to predict the progression of tumor [27]. Alla et al. employed E2F1 knockdown mice to elucidate the effect of E2F1 on skin cancer, and found that endogenous E2F1 deficiency dramatically reduced the migratory and invasive ability of tumors, but the proliferation seemed independent of this change [28]. Recently, the role of E2F1 in small cell lung cancer was also suggested. Li et al. observed the expression of ADAM12, a protein highly expressed in small cell lung cancer, was significantly decreased following silencing of E2F1 by siRNA, which was proved to function via changes in the E2F1 binding to differential cis-acting elements of ADAM1 [29]. In the present study, E2F1 was a candidate of miR-223 targets by TargetScan, which may explain the anti-proliferative function of miR-223. This was further confirmed by luciferase assay, concordantly decreased cell growth rate and E2F1 expression in cells transfected with miR-223. Our study complements the profile of the biological functions of E2F1 in various cell lines.

Apart from the important role in metastasis, E2F1 may also be one of the determinants of chemoresistance [30]. Indeed, Jesper et al. revealed that zebularine-resistant hepatocellular carcinoma cell lines showed up-regulation of key oncogenic networks (TNF, MYC, VHL, and CDKN2A), in which E2F1 was overrepresented, suggesting that their activation by E2F1 may be critical for the development of zebularine resistance [31]. We report a comprehensive characterization of epigenomic modulation caused by zebularine, an effective DNA methylation inhibitor, in human liver cancer. Using transcriptomic and epigenomic profiling, we identified a zebularine response signature that classified liver cancer cell lines into two major subtypes with different drug responses. In drug-sensitive cell lines, zebularine caused inhibition of proliferation coupled with increased apoptosis, whereas drug-resistant cell lines showed up-regulation of oncogenic networks (for example, E2F1, MYC, and TNF). It is reasonable to infer that miR-233, as a regulator of E2F1, may also serve as a mediator of chemoresistance. In the present study, the overexpression of miR-223 sensitized the A549 to gefitinib, which can be inferred from the smaller tumor size of mice injected with cells carrying lentivirus-mediated miR-223. Furthermore, the mice injected with miR-223 overexpressing cells have a higher survival rate than control. This finding is in alignment with a recent study investigating into the enhancement of sensitivity of non-small cell lung cancer cells to erlotinib by miR-223 [32], and our study adds to the jigsaw puzzle of the functional profile of miR-223 in chemoresistance.

In conclusion, the present study hypothesized that miR-223 can inhibit the proliferation and induce apoptosis of NSCLC cells by targeting E2F1. The reduced resistance to gefitinib in miR-223 overexpressing cells demonstrated that miR-223 not only serves as a potential biomarker for prognosis but also an indicator for medication of gefitinib, in which the molecular mechanisms were elucidated by involvement of downregulation of E2F1 in cell proliferation and apoptosis. The survival analysis of mice transfected with lentivirus-mediated miR-223 further supports this hypothesis.

Disclosure of conflict of interest

None.

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References

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