Original Article
MicroRNA 192 regulates chemo-resistance of lung adenocarcinoma for gemcitabine and cisplatin combined therapy by targeting Bcl-2

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Abstract: Lung cancer is the most leading cause of cancer-related death worldwide, with non-small-cell lung cancer (NSCLC) accounting for over 80% of all lung cancer cases. Patients with NSCLC are mostly treated with platinum-based chemotherapy. Chemoresistance is a leading cause of chemo-therapy failure in NSCLC treatment. Recent studies have shown that dysregulation of microRNAs might modulate the resistance of cancer cells to anti-cancer drugs, yet the modulation mechanism is not fully understood. In this paper, we try to test whether miR-192 regulates chemo-resistance in human carcinoma A549 mice model by targeting Bcl-2. Mice model of human lung adenocarcinoma was built up, and was used for gemcitabine and cisplatin combined chemotherapy. MTT assay, real-time RT-PCR, western blotting assay were used to investigate miR-192 expression levels, cell viability ratio and Bcl-2 protein expression levels. MiR-192 expression level in A549 cells is significantly higher than in normal human bronchial epithelial cells. MiR-192 inhibitor treated tumor exhibits sensitivity to cisplatin and gemcitabine therapy. Bcl-2 mRNA and protein expression levels up-regulated in miR-192 inhibitor treated tumor. Bcl-2 is a key regulator for miR-192 related chemotherapy resistance. In this study, we demonstrate that miR-192 regulates chemoresistance for gemcitabine and cisplatin combined chemotherapy in human adenocarcinoma lung cancer A549 cells, and Bcl-2 is the target of miR-192.

Keywords: MicroRNA, chemoresistance, Bcl-2, pathways

Introduction

Lung cancer is the most leading cause of cancer-related death worldwide. Non-small-cell lung cancer (NSCLC) accounts for over 80% of all lung cancer cases [1]. The prognosis for NSCLC remains poor despite advances in the methodologies of diagnosis and chemotherapy. The 5-year survival rate is only 11% [2]. Although Surgery excision is the most effective method for NSCLC treatment, only a few portion of patients can get benefits from it due to the difficulties to diagnose early stage NSCLC. NSCLC patients are often treated with platinum-based chemotherapy. However, patients often develop chemoresistance which leads to chemo-therapy failure [3]. Methods to alleviate chemoresistance are of great interest in NSCLC treatment [4, 5].

MicroRNAs (miRNAs) are short non-coding RNAs that negatively regulate target gene expressions by binding to 3'-untranslated regions of its messenger RNAs leading to degradation or translational suppression [6-8]. Recent studies have shown that miRNAs might modulate chemoresistance of cancer cells although the modulation mechanisms are not clear [9-11]. Several miRNAs were found to promote the NSCLC oncogenesis such as miR-10b, miR-150 and miR-205 [12-14]. On the contrary, miRNA-16, miRNA-140 and miRNA-223 could suppress tumorigenesis [15-17]. MiRNA-192 was associated with oncogenesis of lung cancer, gastric cancer, and colorectum [18, 19]. However, whether miR-192 has any effects on chemoresistance in NSCLC is not known.

The Bcl-2 family proteins play an important role in apoptosis through the balance of anti-apop-
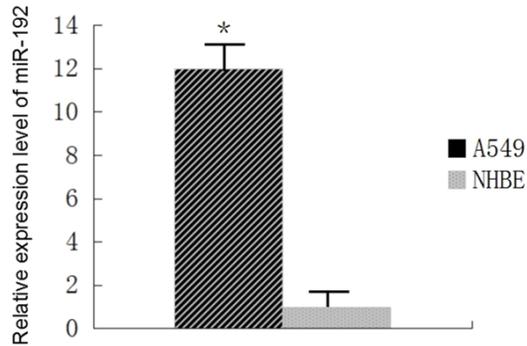
Figure 1. MiR-192 is up-regulated in A549 cells comparing with NHBE. MiR-192 expression levels in A549 and normal human bronchial epithelial cells (NHBE) were compared by Qrt-PCR. The expression levels of miR-192 in A549 cells was significantly higher than in NHBE cells (P < 0.01).

totic proteins (e.g., Bcl-2, Bcl-X_L, Mcl-1) and pro-apoptotic proteins (e.g., Bak, Bax, Bad, Bid) [20]. Bcl-2 proteins are over-expressed in a variety of tumors, which can allow cancer cells escape from apoptosis [21, 22]. Bcl-2 was considered as prognostic impact factor and aggression factor in NSCLC [23, 24]. Whether Bcl-2 is related to chemotherapy drug resistance is unknown. In this paper, we try to test whether miR-192 regulates chemo-resistance in human carcinoma A549 mouse model by targeting Bcl-2.

Materials and methods

Xenograph tumor model in mice

All animal work was approved by the Animal Care Committee of Soochow University in accordance with institutional and Chinese government guidelines for animal experiments. All injections on animals were performed under anesthesia with inhaled isoflurane. Mice were housed in a temperature controlled environment with lights from 06:00-20:00 hour cycle and with water and food freely available. All efforts were made to minimize animal suffering and to reduce experimental animal numbers.

Female Balb/c nude mice (3 months old, 180-220 grams) were purchased from Shanghai SLAC Laboratory Animal Co., LTD (Shanghai, China). Mice were housed singly in polyethylene cages with hardwood-chip bedding and given free access to food and water. Five million A549 cells were subcutaneously injected in the right flank of 25 mice with inhaled isoflurane.

Mice body weights and tumor growth rates were monitored twice a week for 3 weeks. The tumor volume was calculated with the formula (long dimension) × (short dimension)^2/2. Two mice died within one week after the study started. Only 18 mice carrying tumors reached approximately 200 mm^3, and body weights ranged from 160-240 grams were included into this study. Five mice were sacrificed with inhaling carbon monoxide in their home cage due to either too low body weights or tumor sizes are too big. These 18 mice were randomly divided into two groups (n = 9 mice/each group), and treated with either vehicle or gemcitabine plus cisplatin by tail vein injection. The dose and timetable of injection was shown in **Figure 4A**. The body weight and survival of the nude mice were monitored twice per week throughout the experiments. The mice were sacrificed by decapitation to collect tumor samples for further analysis.

Cell culture

The human lung adenocarcinoma cell line A549 was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Normal human bronchial epithelial cells (NHBE) (CloneticsTM) were maintained in a culture medium according to the protocol provided by CloneticsTM. A549 cell line was cultured in DMEM containing 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator.

Drugs and reagents

Cisplatin was purchased from QiLu Pharmaceutical (Jinan, China). Gemcitabine was purchased from HaoSeng Pharmaceutical (Jiangsu, China). MiR-192 inhibitor, Bcl-2 siRNA, and their negative control oligonucleotides were obtained from Invitrogen (Carlsbad, CA, USA). These were used to transfect A549 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the manufacture.

Western blot

The proteins were loaded onto a 4% SDS denaturing polyacrylamide stacking gel, and separated on a 10% SDS denaturing polyacrylamide running gel, then transferred onto a nitro-
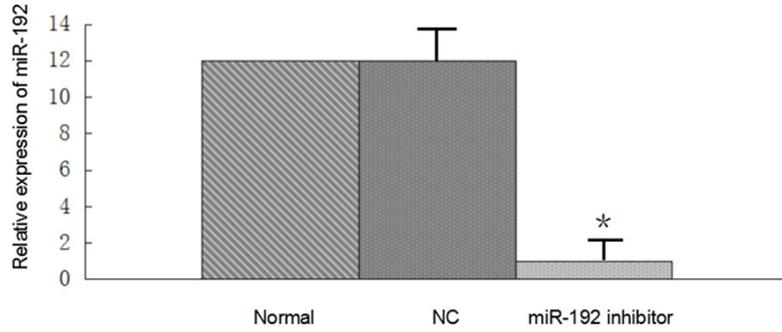
Figure 2. MiR-192 inhibitor suppress miR-192 expression levels in A549 cells. MiR-192 inhibitor and its negative control oligonucleotides (NC) into A549 cells. Transfection with miR192 inhibitor suppressed miR-192 expression level compared with the negative control group (P < 0.01). This result suggests that miR-192 inhibitor works properly to suppress miR-192 expression in A549 cells.
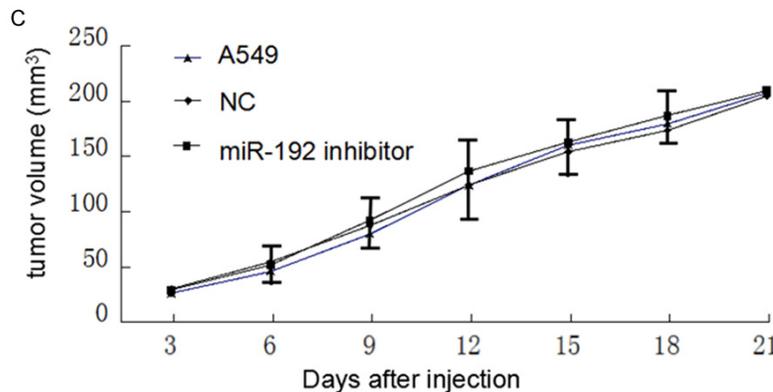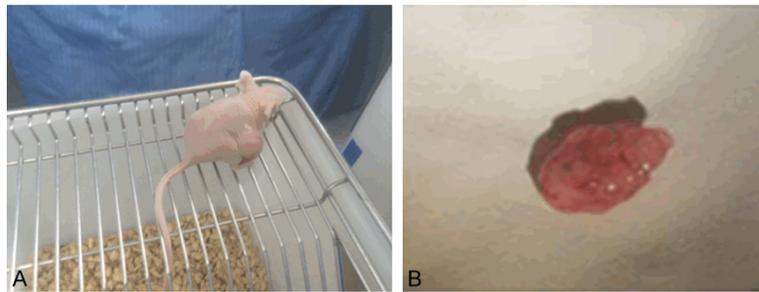
Figure 3. MiR-192 is not involved in human lung adenocarcinoma tumorigenesis. A. Shows that Balb/c mice carried human lung tumor; B. Shows the tumor tissue after dissected from its host; C. Shows that tumor growth rates did not show significant difference between miR-192 inhibitor treated and not treated groups (P > 0.05). NC stands for negative control of miR-192 inhibitor.

cellulose membrane. Antibody to Bcl-2 and GAPDH was incubated overnight at 4°C (Abcam, Cambridge, MA, USA). The membrane was washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge, MA, USA). Protein expression was assessed by enhanced chemiluminescence and exposure to chemiluminescent film.

Quantitative RT-PCR

RNAs were collected from 1 mm³ cancer tissue. Total RNAs were extracted using RNeasy columns (Qiagen) according to the manufacture instructions. Total RNA (1 g) was reverse transcribed into cDNA (Clontech). SYBR green real-time PCR kit (Applied Biosystems) was used to quantify gene expression levels. Data were analyzed with SDS Relative Quantification Software version 2.2.2 (Applied Biosystems). Ct values were exported into Excel software for data analysis. miR192 forward primer is: GTGGACCTGACCTGCCGTCT; miR192 reverse primer is: GGAGGAGTGGGTGTCGCTGT. GAPDH forward primer is: AAGGGAAGGTTGCTGGATAGG; GAPDH reverse primer: CACATCCACCTCCTCCACATC.

MTT assay

Cells transfected with miR-192 inhibitor or siRNA-Bcl2 were seeded into 96-well plates at 6000 cells per well. After growing overnight, cells were treated with different concentrations of cisplatin and gemcitabine. After 24 hours of treatment, 20 ml of 5 mg/ml MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated in the dark for 4 hours. The absorbance of the plate was measured in a microplate reader at a wavelength of a 570-nm reference. Each treatment was carried out in triplicate.

Statistical analysis

The data from the experiments at different time points for the different treatment groups were
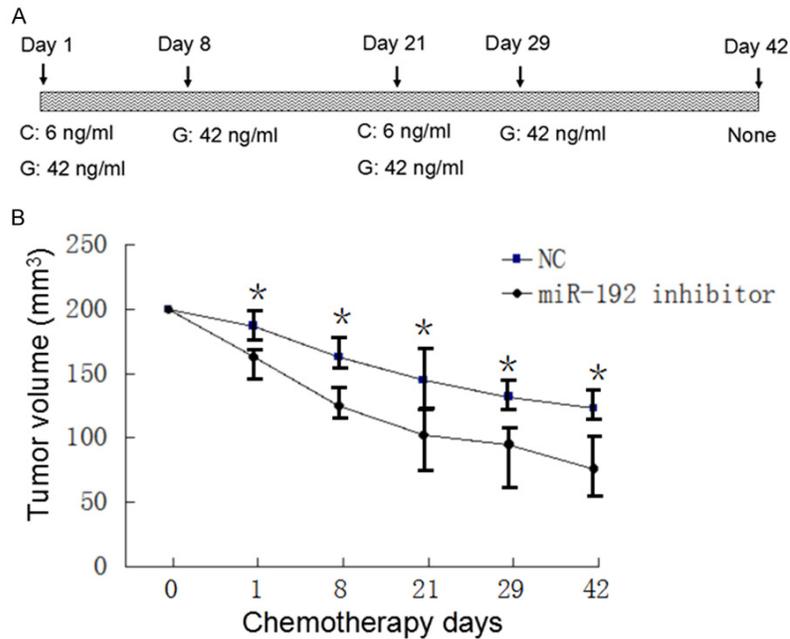
Figure 4. MiR-192 inhibitor treated tumor exhibits sensitivity to ciaplatin and gemcitabine therapy. A. Demonstrates timetable and drug doses of Gemcitabine and cisplatin combined chemotherapy. B. Shows tumor sizes shrank during chemotherapy. MiR-192 treated human lung cancer tumor decreased significantly comparing with negative control group (P < 0.05).

analysed for statistical significance with the SPSS 13.0 statistical software, analysis among different groups used One-way ANOVA, T test was used to compare two different groups. P < 0.05 was considered as significance.

Results

MiR-192 expression level in A549 cells is significantly higher than in NHBE cells

MiR-192 expression levels in A549 and normal human bronchial epithelial cells (NHBE) were compared by qRT-PCR. The expression levels of miR-192 in A549 cells was significantly higher than in NHBE cells (P < 0.01, **Figure 1**). This result suggests that miR-192 may have a role in human lung cancer tumorigenesis.

MiR-192 inhibitor suppress miR-192 expression levels in A549 cells

We transfected miR-192 inhibitor and its negative control oligonucleotides into A549 cells. Transfection with miR192 inhibitor suppressed miR-192 expression level compared with the control group (**Figure 2**). This result suggests that miR-192 inhibitor works properly to sup-

press miR-192 expression in A549 cells.

MiR-192 is not involved in human lung adenocarcinoma tumorigenesis

A549 cells without any treatment, A549 cells with miR-192 inhibitor treatment, and with miR-192 inhibitor negative control treatment cells were subcutaneously injected in the right flank of 5 weeks old Balb/c female mice. Tumor growth was monitored by measuring the tumor size twice a week for 3 weeks after treatment. The tumor volume was calculated with the formula (long dimension) × (short dimension)^2/ 2. **Figure 3A** and **3B** show the tumor size around 200 mm^3. Sizes and volumes of tumors did not show significant difference with or without miR-192 inhibitor treatment (P > 0.05, **Figure 3C**). This result suggests that miR-192 is not involved in human lung adenocarcinoma tumorigenesis.
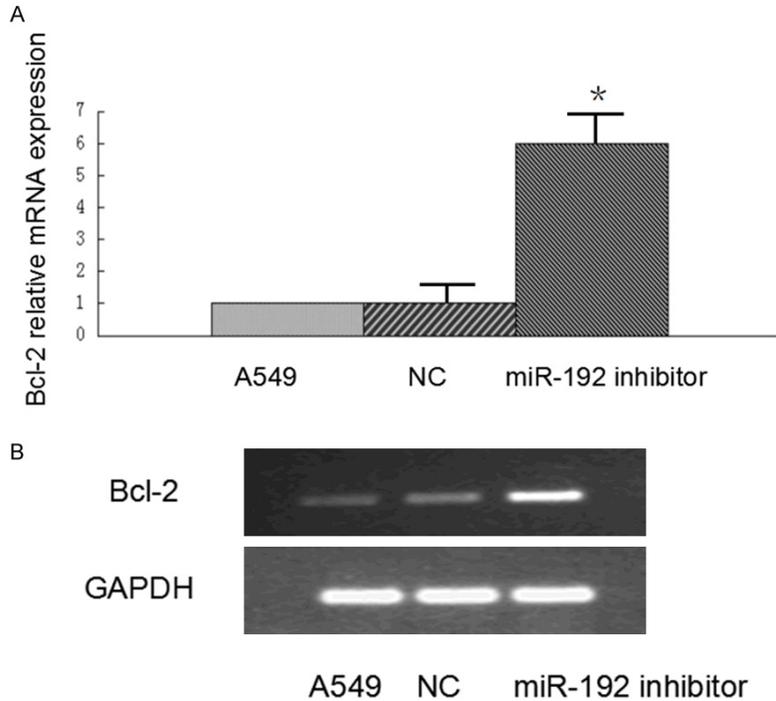
MiR-192 inhibitor treated tumor exhibits sensitivity to cisplatin and gemcitabine therapy

The mice carried miR-192 inhibitor treated tumor or miR-192 inhibitor negative control nucleotide treated tumor were treated with gemcitabine and cisplatin combined chemotherapy. Cisplatin concentration range was 6 ng/ml. Gemcitabine concentration was 42 ng/ml. Detailed chemotherapy timetable was shown in **Figure 4A**. Tumor sizes were measured every week. The results showed that miR-192 inhibitor treated tumor was more sensitive to chemotherapy comparing with negative control group (P < 0.05, **Figure 4B**).

Bcl-2 mRNA and protein expression levels up-regulated in miR-192 inhibitor treated tumor

Total RNAs were extracted from miR-192 inhibitor treated tumor and miR-192 inhibitor negative control treated tumor tissues. Bcl-2 mRNA level was over-expressed in miR-192-suppress-

Figure 5. Bcl-2 mRNA and protein expression levels up-regulated in miR-192 inhibitor treated tumor. Total RNAs were extracted from miR-192 inhibitor treated tumor and miR-192 inhibitor negative control treated tumor tissues. Bcl-2 mRNA level was over-expressed in miR-192-suppressed tumor compared with controls (P < 0.01, A). Bcl-2 protein expression level was up-regulated compared with controls (B).
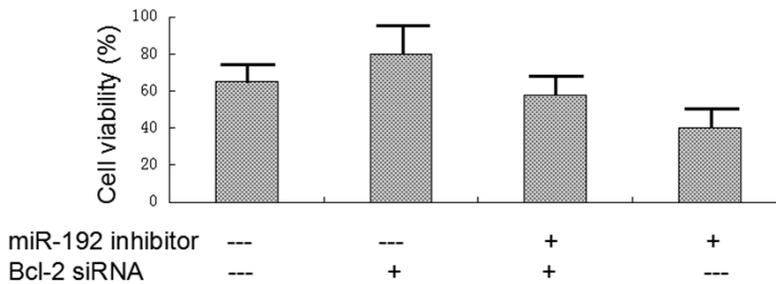
Figure 6. Bcl-2 is a key regulator for miR-192 related chemotherapy resistance. A549 cells were transfected with Bcl-2 siRNA, miR-192 inhibitor or both. MTT assay was performed, cells were treated with gemcitabine combined with cisplatin overnight. The lowest cell viability was from miR-192 inhibitor transfected cells, the highest cell viability was from Bcl-2 siRNA transfected cells. Co-transfected cells viability was slightly lower than Bcl-2 siRNA transfected cells. These result suggest that Bcl-2 is a key regulator for miR-192 related resistance for gemcitabine and cisplatin combined chemotherapy.

ed tumor compared with controls (**Figure 5A**). During chemotherapy, Bcl-2 mRNA levels were over-expressed compared with controls (data not shown). Bcl-2 protein expression level was up-regulated compared with controls (**Figure 5B**). These suggest that Bcl-2 is a target of miR-192.

Bcl-2 is a key regulator for miR-192 related chemotherapy resistance

MTT assay was used to test whether Bcl-2 is responsible for miR-192 related chemotherapy resistance. A549 cells were transfected with Bcl-2 siRNA, miR-192 inhibitor or both. MTT assay was performed, cells were treated with gemcitabine combined with cisplatin overnight. The lowest cell viability was from miR-192 inhibitor transfected cells, the highest cell viability was from Bcl-2 siRNA transfected cells. Co-transfected cells viability was slightly lower than Bcl-2 siRNA transfected cells (**Figure 6**). These results suggest that Bcl-2 is a key regulator for miR-192 related resistance for gemcitabine and cisplatin combined chemotherapy.

Discussion

The efficacy of chemotherapeutic agents is often limited by chemoresistance in the treatment of NSCLC. Gemcitabine is a nucleoside analog in which the hydrogen atoms on the 2' carbon of deoxycytidine are replaced by fluorine atoms [25]. The triphosphate analogue of gemcitabine replaces one of the building blocks of cytidine during DNA replication. The process arrests tumor growth, resulting in apoptosis. Cisplatin is a platinum-based compound that forms intra- and inter-strand addicts with DNA [26, 27]. Gemcitabine combined with cisplatin is widely used in the treatment of NSCLC despite of development of drug resistance. The molecular mechanisms leading to chemoresistance for gemcitabine plus cisplatin therapy are widely unknown.

MiRNAs are considered as oncogene or tumor suppressors [28-30]. Different miRNA has variant effects during cancer development. MiR-192 was considered as tumor suppressor in human chondrosarcoma [31], and involved in tumor blood vessel development, therefore it worked as oncogene [32]. The Bcl-2 family proteins play an important role in apoptosis. Our study demonstrated that miR-192 regulates chemoresistance to gemcitabine and cisplatin in A549 cells, and Bcl-2 is the target of miR-192. However, miR-192 is not involved in NSCLC tumorogeneis in this mouse human disease model. Whether miR-192 could be considered as chemosensitivity biomarker needs further investigations.

Previous studies demonstrated that deregulation of miRNAs such as miR-21, miR-503, miR-181a and miR-620 is related to drug resistance [33-35]. Many miRNAs and oncogene target pathways, such as miR7/Bcl2, miR-99b/FGF3, and miR196/HOXA5 have been demonstrated to participate in the tumorigenesis of lung cancer [36-38]. Whether miR-192 has function through other pathways other than Bcl-2 pathways need to be investigated. It is reasonable expectation that chemoresistance is related with apoptosis, which had never been reported before. We need to study chemoresistance of other lung cancer types besides NSCLC to further study this possible linkage.

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Disclosure of conflict of interest

None.

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