Modulation of NF-κB/miR-21/PTEN Pathway Sensitizes Non-Small Cell Lung Cancer to Cisplatin

Zhenhua Yang, Surong Fang, Yicheng Di, Weiwei Ying, Yan Tan, Wei Gu*
Department of Respiratory Medicine, Nanjing First Hospital, Nanjing Medical University, Nanjing, China
* njguwei@163.com

Abstract

Background
Platinum-based chemotherapy is a standard strategy for non-small cell lung cancer (NSCLC), while chemoresistance remains a major therapeutic challenge in current clinical practice. Our present study was aimed to determine whether inhibition of the NF-κB/miR-21/PTEN pathway could increase the sensitivity of NSCLC to cisplatin.

Methods
The expression of miR-21 in NSCLC tissues was determined using in situ hybridization. Next, the effect of miR-21 on the sensitivity of A549 cells to cisplatin was determined in vitro. Whether miR-21 regulated PTEN expression was assessed by luciferase assay. Furthermore, whether NF-κB targeted its binding elements in the miR-21 gene promoter was determined by luciferase and ChIP assay. Finally, we measured the cell viability and apoptosis under cisplatin treatment when NF-κB was inhibited.

Results
An elevated level of miR-21 was observed in NSCLC lung tissues and was related to a short survival time. Exogenous miR-21 promoted cell survival when exposed to cisplatin, while miR-21 inhibition could reverse this process. The RNA and protein levels of PTEN were significantly decreased by exogenous miR-21, and the 3’-untranslated region of PTEN was shown to be a target of miR-21. The expression of miR-21 was regulated by NF-κB binding to its element in the promoter, a finding that was verified by luciferase and ChIP assay. Hence, inhibition of NF-κB by RNA silencing protects cells against cisplatin via decreasing miR-21 expression.

Conclusion
Modulation of the NF-κB/miR-21/PTEN pathway in NSCLC showed that inhibition of this pathway may increase cisplatin sensitivity.
Introduction
Non-small cell lung cancer (NSCLC), comprising squamous cell carcinoma, adenocarcinoma and large cell undifferentiated carcinoma, is the most common type of lung cancer [1,2]. Genetics plays an essential role in the pathophysiological mechanism of NSCLC [3,4], and it leads to non-sensitivity of NSCLC to platinum-based chemotherapy [5], resulting in NSCLC being the most common cause of cancer-related mortality worldwide [1]. Thus, it is necessary to develop novel drug targets based on molecular genetics.

MicroRNAs (miRNA) have been implicated as key modulators of multiple target genes through the endogenous RNA interference machinery [6]. They contribute to cancer biology by altering the expression of their target genes [7]. miR-21 is one type of miRNA that functions as a potent modulator of tumor cell behavior and malignant transformation [8]. It has been found to increase cell growth in liver cancer and has demonstrated anti-apoptotic properties in glioblastoma [9]. In NSCLC, miR-21 also plays a fundamental role in cellular proliferation, invasion and apoptosis [10]. Previous studies have demonstrated that miR-21 regulates the expression of phosphatase and tensin homolog (PTEN) via directly targeting its 3’ untranslated region (3’UTR) [8]. PTEN, a tumor suppressor gene, plays an essential role in the regulation of the cell cycle, apoptosis and formation of many types of solid tumors, including NSCLC [10]. It negatively regulates the intracellular levels of phosphatidylinositol-3,4,5-trisphosphate (PI3K) in cells and functions as a tumor suppressor by negatively regulating the Akt signaling pathway [11]. Under normal conditions, the expression and activation of PTEN is tightly controlled, whereas the expressions of miRNAs are dysregulated in NSCLC. Alteration of miR-21/PTEN was found in NSCLC patients with gefitinib resistance [12].

Given the essential role of the miR-21/PTEN pathway in NSCLC, there remains a question regarding why the expression of miR-21 is increased in NSCLC. We performed a bioinformatics search (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promo.cgi) and found four binding elements of NF-κB in the promoter of the miR-21 gene. Additionally, one research group demonstrated that DNA damage induced miR-21 upregulation and promoted breast cancer cell invasion in an NF-κB-dependent manner [13]. Hence, we assumed that NF-κB increased the level of miR-21, which reduced PTEN expression post-transcriptionally to promote cell survival under cisplatin treatment in NSCLC; therefore, inhibition of the NF-κB/miR-21/PTEN pathway might be a potential drug target for NSCLC.

Methods
Tissue microarray
Tissue microarray (TMA) was prepared from tissues derived from 34 patients with NSCLC and 10 patients without NSCLC between January 1999 and August 2007. The study was conducted in accordance with the Declaration of Helsinki (1989) and was approved by the local Ethics Committee (Nanjing First Hospital, Nanjing Medical University, Nanjing, China). Written informed consent was obtained from each patient. The representative area was carefully selected from a hematoxylin and eosin (HE)-stained section, and then a 1.5 mm tissue core was obtained from the corresponding paraffin blocks. Thereafter, paraffin-embedded material was cut into 5 μm sections and placed onto a slide, followed by staining with HE or in situ hybridization (ISH). Sections were viewed under a light microscope (Olympus, Japan). NSCLC was diagnosed by two independent pathologists according to the Union for International Cancer Control (UICC) classification system, 7th edition. The staining intensity was scored as follows: 0, no staining of cells; 1, weak staining; and 2, strong staining. The percentage of stained cells...
was classified using a 3-grade scale: 0, no positive cells; 1, <50% positive cells; and 2, >50% positive cells.

**ISH**

ISH of miR-21 was performed according to the manufacturer’s protocol (MK10013, Boster, China). Briefly, after deparaffinization of slides in xylene and ethanol, slides were incubated with 3% H$_2$O$_2$ for 10 min at room temperature and then digested with pepsin for 10 min at 37°C. After rinsing in water, slides were fixed with 1% PFA in DEPC (Generay, China) for 10 min. Sections were then washed in water three times and incubated with prehybridization buffer for 4 h at 42°C and hybridization buffer overnight at 42°C with an miR-21 probe (5'-TAGCTTATCAGACTGATGTTGA-3'). Slides were washed as follows: 2×SSC for 5 min at 37°C, twice; 0.5×SSC for 15 min at 37°C, once; and 0.2×SSC for 15 min at 37°C, once. The tissue was incubated in blocking buffer at 37°C for 30 min and then was incubated with streptavidin-biotin complex (SABC). After washing three times with PBS, slides were incubated with horseradish peroxidase polymer conjugate for an additional 30 min at room temperature. Subsequently, they were stained with 3,3-diaminobenzidine and counterstained with hematoxylin (Sigma-Aldrich, US).

**Cell culture**

HEK293 cells and A549 cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, US) supplemented with 10% fetal bovine serum (FBS, Gibco, US), penicillin 100 U/ml and streptomycin 100 μg/ml (Gibco).

**Construction of plasmid and recombinant lentivirus**

The human NF-κB (p65) gene fragment amplified by PCR was cloned into pcDNA3 and the insertion was confirmed by sequencing. Then, the plasmid pcDNA-NF-κB was digested by EcoRI/XbaI. The digested products were electrophoresed in an agarose gel. The NF-κB gene fragment was purified by gel extraction and subsequently cloned into pLVX-IRWS-ZsGreen1. An shRNA construct targeting NF-κB was also cloned into pLVX-IRWS-ZsGreen1. Recombinant lentiviruses Lenti-shNF-κB and Lenti-NF-κB were produced and titrated according to a previous report [14]. The entire open reading frame of PTEN with or without the miR-21 targeting sequence in the 3'UTR was cloned into the pcCS2 vector with a Myc tag to construct pcCS2-PTEN-3'UTR and pcCS2-PTEN, respectively.

**Transfection of miR-21 mimics and inhibitor**

The FAM-modified 2'-O-Me-oligonucleotides were chemically synthesized and purified by high-performance liquid chromatography (GenePharma, China). The 2'-O-Me-miR-21 mimic was composed of RNA duplexes with the following sequence: 5'-UAGCUUAUCAGACUGAU GUUGA-3'; the sequences of 2'-O-me-miR-21 inhibitor and 2'-O-me-scrambled oligonucleotides were as follows: 5'-UCAACAUCAGACUGAUAAACUA-3'; and 5'-CAGUACUUUUGUGUA GUACAA-3'. The cells were seeded on a 24-well plate at a density of 5×10^4 cells per well and grown to 80% confluence. The miR-21 inhibitor or miR-21 mimics at a concentration of 50 nM each was transfected into cells using Lipofectamine 2000 (Invitrogen, USA) by incubation in Opti-Mem I media (Invitrogen) for 4 h. The cells were then transferred into fresh culture medium. After incubation for 24 h, the culture medium was replaced and fluorescent images were acquired to monitor the transfection efficiency. After 48 h, cells were harvested for analysis.
Real-time PCR

Total RNA was isolated from cells using Trizol reagent (Invitrogen) following the manufacturer’s recommendation. For miR-21 qRT-PCR, total RNA was reverse transcribed using an miRNA-specific primer and the miScript Reverse Transcription kit (Qiagen, Germany). Stem-loop qRT-PCR was performed using the SYBR green PCR Master Mix (Qiagen) according to the manufacturer’s protocol. Relative expression was evaluated by the comparative Ct method and normalized to the expression of U6 small nuclear RNA. For the detection of PTEN, reverse transcription was carried out using the First-strand cDNA synthesis kit (Promega, US) following the manufacturer’s recommendations. qRT-PCR was performed using the QuantiFast SYBR Green PCR Kit (Qiagen). The GAPDH mRNA level was employed as an internal control. All of the samples were analyzed in triplicate in three independent experiments. Reactions without cDNA were used as the no-template control, and no-RT controls were also set up to exclude genomic DNA contamination. The relative quantification of mRNA expression was determined using the comparative Ct method.

Western blotting

Cells were homogenized in RIPA buffer (Cell-Signaling Tech., US). The protein concentrations were determined using a BCA kit (Beyotime, China). Equal amounts of protein were separated on SDS-PAGE and then transferred to 0.45-μm PVDF membranes (Bio-Rad, US). The membranes were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 buffer (TBST) for 2 h and then incubated overnight at 4°C with the following primary antibodies: anti-PTEN (1:1000, Cell-signaling Tech.), anti-total Akt (1:1000, Abcam), anti-phospho ser473 Akt (1:1000, Abcam) and anti-GAPDH (1:1000, Cell-signaling Tech.), which served as a loading control. Next, the membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG (1:5000) or goat anti-mouse IgG (1:5000); Cell-signaling Tech.) for 2 h at room temperature. Blots were developed using a chemiluminescence kit (Millipore, US) and exposed to X-ray film. The bands on the film were scanned and analyzed using Image J.

Luciferase vector construction

The human PTEN 3’UTR containing the seed sequence of the miR-21 binding sites was amplified by PCR and was cloned into the pMIR-REPORT vector (Ambion, USA) between the HindIII and SpeI sites to develop the pMIR-PTEN-3’-UTR luciferase vector. Seed sequences were mutated by overlap extension PCR. The mutated PTEN-3’-UTR fragment was cloned into the pMIR-REPORT vector to develop the pMIR-PTEN-mut-3’-UTR vector. A series of reporter constructs with the same 3’ terminus but different 5’ terminus of the miR-21 promoter were amplified by PCR. All of the PCR products were cloned into the plasmid pGL4-Minimal (Promega) to generate pGL4-miR-21. The mutant site was introduced into the plasmid by overlap extension PCR. The subsequent PCR product was cloned to generate pGL4-mut-miR-21. All of the constructs were confirmed by DNA sequencing.

Luciferase activity

Cells at a density of 2×10^5 per well were seeded into 24-well plates and cultured overnight. For the miR-21 assay, cells were cotransfected with 0.8 μg of pMIR-PTEN-3’-UTR or pMIR-PTEN-mut-3’-UTR, 50 nM miR-21 mimic or inhibitor or scrambled oligonucleotide along with 50 ng of pRL-TK vector as an internal control using Lipofectamine 2000 reagent (Invitrogen). For the promoter assay, cells were co-transfected with 1 μg of pcNF-κB or pcDNA, 20 ng of a
luciferase reporter plasmid and 10 ng of the Renilla luciferase reporter plasmid (Promega). Twenty-four hours after transfection, the cells were harvested, and the luciferase activity was determined using a dual luciferase reporter assay (Promega, USA). The results were expressed as relative luciferase activity as reported previously [15]. All experiments were repeated three times in triplicate.

**ChIP assay**

The ChIP assay was performed using the Magna ChIP A/G kit (Millipore) according to the manufacturer’s recommendation. Briefly, the A549 cells were cross-linked with 1% formaldehyde (Sigma-Aldrich) for 10 min and quenched by glycine. The cross-linked cells were collected in cold PBS and sonicated for six 15-s pulses at 50-s intervals to reduce the total DNA size to 200–1000 bp. The sheared chromatin and magnetic beads were immunoprecipitated with anti-NF-κB (ab7970; Abcam, USA) or normal rabbit IgG overnight at 4°C on a rotator. Magnetic bead-antibody-chromatin complexes were washed once with a low-salt buffer, followed sequentially by high salt, LiCl and TE buffers. The chromatin complexes were eluted and incubated at 62°C for 2 h. The DNA samples were recovered using spin columns. For ChIP samples, real-time PCR was carried out using the SYBR method as described above.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay**

Cells were seeded into 96-well plates at a density of 5000 cells per well. After 24 h, cells were transfected with 100 nM miR-21 mimic, miR-21 inhibitor or scrambled oligonucleotide. For combination experiments with the miR-21 mimic and pcDNA-PTEN, cells were cotransfected with pcDNA-PTEN (0.2 μg). The medium was replaced with fresh complete medium with or without cisplatin (5 μM) at 24 h post-transfection according to a previous report [16]. After an additional 24 h, 20 μl of 5 mg/mL MTT (dimethyl thiazolyl diphenyl tetrazolium; Sigma-Aldrich) was added into each well and incubated for 4 h in a humidified incubator. The supernatant was then discarded, and 200 μL of DMSO was added to each well to dissolve the formazan. The optical density (OD) was measured at 490 nm. The viability of the cells without any treatment was defined as 100%, and the viability of other groups was calculated separately from that of the sham cells.

**Statistics**

Continuous variables were presented as the mean ± SD and determined using analysis of variance (ANOVA) followed by a post-hoc t-test using SPSS 13.0 software. The association between survival rate and miR-21 expression was determined using multiple logistic regression analysis including the following factors: age, gender, smoker and histology. Kaplan-Meier analysis was used to evaluate the rate of event-free survival and the primary patency. Statistical significance was defined as a P value less than 0.05.

**Results**

**miR-21 is overexpressed in NSCLC lung tissues**

Lung tissues from 34 patients with NSCLC and 10 patients without NSCLC were used to make the TMA. The clinical characteristics of the subjects are shown in Table 1. The expression of miR-21 was detected by ISH. The intensity of miR-21 staining was significantly increased in NSCLC cells. The score was significantly increased in patients with NSCLC (Fig. 1A and B;
Moreover, the survival time was decreased in patients with NSCLC who had a high level of miR-21 compared to those who had a low level of miR-21 (Fig. 1C; P < 0.05).

miR-21 modulates the survival of A549 cells

The miR-21 mimics were used to represent exogenous miR-21, and the miR-21 inhibitor was used to inhibit endogenous miR-21. The level of miR-21 in the A549 cells was detected by real-time PCR (Fig. 2B). Next, we used the MTT assay to evaluate the cell viability of A549 cells when exposed to cisplatin treatment. As shown in Fig. 2C, the cell viability was significantly increased in cells treated by miR-21 mimics compared to the control group. Inhibition of miR-21 by its inhibitor decreased the cell viability compared to the control group.

Table 1. Demographic and clinical characteristics.

|                          | Overexpressed miR-21 | Non-overexpressed miR-21 | Non-NSCLC |
|--------------------------|-----------------------|--------------------------|-----------|
| Age, y                   | 64                    | 65                       | 64        |
| Male                     | 8/17                  | 9/17                     | 5/10      |
| Smoker                   | 13/17                 | 15/17                    | 8/10      |
| Histology                |                        |                          |           |
| Adenocarcinoma           | 9                     | 9                        | -         |
| Squamous carcinoma       | 3                     | 4                        | -         |
| Bronchioloalveolar       | 5                     | 4                        | -         |
| carcinoma                |                       |                          |           |

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Fig 1. Overexpression of miR-21 in NSCLC patients. (A) ISH was used to detect the miR-21 in a TMA with 34 NSCLC and 10 normal lung samples. Left panel was ISH and right panel was HE staining. (B) The staining intensity score was higher in NSCLC patients compared with normal subjects. (C) The survival time of patients with high level of miR-21 was shorter than who with low level of miR-21. *P < 0.05 as significant difference.

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miR-21 regulates PTEN expression by binding to its 3'-UTR

A bioinformatics search demonstrated that PTEN, a tumor-suppressor gene, was a potential target for miR-21 because there was a putative miR-21-binding seed sequence within the 3’UTR of PTEN mRNA (Fig. 3A). We measured the mRNA (Fig. 2B) and protein levels of PTEN (Fig. 2D) in the A549 cells with a high or low level of miR-21. An inverse correlation between miR-21 expression and PTEN mRNA expression was detected. Akt is downstream of PTEN, and I also affected the activation of Akt (Fig. 2D and E). Compared to the control group, miR-21 mimics decreased the luciferase activity, whereas the miR-21 inhibitor showed a significant increase in the relative luciferase activity. When the seed sequence of the miR-21 binding sites was mutated, the effect of miR-21 on luciferase activity was not detected (Fig. 3B). Next, the entire open reading frame of PTEN with or without the miR-21 targeting sequence in the 3’UTR was cloned into pcCS2. The Myc level, which represented exogenous PTEN, was decreased by the miR-21 mimics in the cells transfected with pcCS2-PTEN-3’UTR, whereas there
was no significant reduction in Myc expression by miR-21 mimics in the cells transfected with pcCS2-PTEN (Fig. 3C and D). These results implied that miR-21 regulated the expression of PTEN in A549 cells via targeting the sequence of the PTEN 3'UTR.

NF-κB regulates the expression of miR-21 via binding its promoter

Given the role of miR-21 in NSCLC, we aimed to investigate the mechanism of overexpression of miR-21 in NSCLC. The bioinformatics analysis suggested four potential binding elements of NF-κB in the promoter region of the miR-21 gene (Fig. 4A). The ChIP real-time assay showed that the level of NF-κB antibody binding to the miR-21 promoter was more than that of IgG (Fig. 4C, P<0.05), indicating that NF-κB could directly bind to all four elements in the promoter region of the miR-21 gene. Next, we subcloned different lengths of the miR-21 promoter and its mutation into pGL4 and cotransfected the constructs with Renilla with or without pcNF-κB into A549 cells. As shown in Fig. 4B, pcNF-κB significantly increased the luciferase activity compared to pcDNA. When the first binding element (-2837) was deleted or mutated, there was no reduction in luciferase activity compared to the full-length promoter. When the second element (-1211) was deleted or mutated, there was significant reduction in luciferase activity. However, when the binding sequences of NF-κB were all deleted or mutated, there was also significant reduction in luciferase activity compared to when the second element was deleted or mutated. These results
indicated that the second and fourth binding elements might contribute to the regulation of miR-21 transcription by NF-κB.

The NF-κB/miR-21/PTEN pathway modulates the sensitivity of A549 cells to cisplatin

The level of miR-21 was induced by overexpression of NF-κB and inhibited by Lenti-shNF-κB, which decreased the expression of NF-κB (Fig. 5A). PTEN expression was inversely correlated with NF-κB expression (Fig. 5A). The cell viability of A549 cells was significantly increased by Lenti-NF-κB when exposed to cisplatin treatment (Fig. 5B). In the cells transfected with pcCS2-PTEN to overexpress PTEN, the effects of overexpression of NF-κB or miR-21 on the cell viability of A549 cells was inhibited (Fig. 5C).

Discussion

In the present study, we demonstrated that miR-21 was upregulated in patients with NSCLC, indicating a poor prognosis. Additionally, it was found that miR-21 induced the resistance of the NSCLC cell line to cisplatin via targeting the 3'UTR of PTEN to decrease its expression post-transcriptionally. Moreover, NF-κB translocates into the nucleus and binds to its specific elements in the miR-21 gene promoter to induce miR-21 expression. Finally, modulation of the NF-κB/miR-21/PTEN pathway increased the sensitivity of NSCLC cells to cisplatin treatment.
Our data might provide new insights into the pathophysiological mechanism of NSCLC and suggests a novel drug target for NSCLC.

miR-21 has been found to be overexpressed in many types of solid tumors, including liver cancer, prostate cancer and colorectal cancer. In our present study, we extracted tissues from patients with NSCLC and control subjects to perform TMA. The ISH result detected a higher level of miR-21 in patients with NSCLC compared to control subjects. Furthermore, the high level of miR-21 indicated a poor prognosis for patients with NSCLC. This is supported by many previous studies. In the Markou et al. study, 48 NSCLC fresh-frozen tissue specimens were collected to detect miR-21 expression by real-time PCR, and it was found that miR-21 was upregulated in 16 of 29 (55.2%) relapsed patients and 15 of 23 (65.2%) deceased patients [17]. Additionally, in non-smokers, the aberrantly increased expression of miR-21 was found to contribute to lung carcinogenesis [18]. Two meta-analyses summarizes the data from different studies and concluded that overexpression of miR-21 was an independent prognostic factor for NSCLC [19], particularly in Asian people [20].

To understand the effect of miR-21 on NSCLC, miR-21 mimics and its inhibitor were used, respectively, to enhance and inhibit its biological function in the A549 NSCLC cell line. It was found that exogenous miR-21 promoted cell survival when exposed to cisplatin treatment, whereas miR-21 inhibition led to the opposite function. These findings were validated by one study, which showed that elevated miR-21 increased the resistance of A549 cells to platinum, whereas reduced miR-21 decreased this resistance [21]. Based on the above findings, miR-21 might be a potential therapeutic target for NSCLC. The underlying mechanism of cisplatin resistance of NSCLC is complicated, and miR-21 also has multiple targets according to the bioinformatics analysis. To explore the effect of I on NSCLC before it advances further into the clinical application stage, two essential questions persist: how I affects the outcome of NSCLC, and how I is upregulated in NSCLC.

Bioinformatics studies suggest a target site of miR-21 in the 3’UTR of PTEN mRNA. I is a ubiquitous tumor-suppressor gene, and its deregulation results in many solid tumors. In NSCLC, I has been implicated as a key contributor to pathogenesis and tumor growth [22]. PTEN loss has been recognized as the mechanism of gefitinib resistance in NSCLC [23]. Targeted deletion of PTEN leads to the development of NSCLC [24]. Hence, it was assumed that
miR-21, which inhibits PTEN by targeting its 3'UTR, would affect the NSCLC pathological process. We initially identified an inverse relationship between miR-21 and PTEN in A549 cells. We then cloned the 3'UTR of PTEN into the pMIR-REPORT luciferase reporter and found that miR-21 mimics significantly decreased luciferase activity when cotransfected with pMIR-REPORT-PTEN-3'UTR, whereas no change in luciferase activity was noted when miR-21 mimics were cotransfected with pMIR-REPORT-PTEN without the 3'UTR. Previous studies have also supported this finding [25,26].

On one hand, miR-21 regulated the expression of PTEN; on the other hand, it was regulated by other factors. Some miRNAs have their own promoters, whereas others share a promoter with other miRNAs for the miRNA gene located in the intron of the host gene. Whether miRNAs have their own promoters or share promoters, their expression can be regulated by certain transcription factors, including p53 and NF-κB, which target their promoters. There are four binding elements of NF-κB in the miR-21 gene promoter [27]. Hence, it is possible that miR-21 could be regulated by NF-κB targeting its binding elements. Our ChIP assay confirmed that NF-κB could directly bind to all four elements in the promoter of the miR-21 gene. However, only the elements located at -1211 and -404 of the miR-21 gene promoter had biological function according to the luciferase activity assay. Our data were partially supported by those of other studies. In prostate cancer cells, the interferon-induced miR-21 expression required NF-κB/p65 recruitment to the miR-21 promoter [27]. In the human breast MDA-MB-231 and colorectal HCT116 tumor cell lines, NF-κB was also found to bind to the miR-21 gene promoter [28]. In NSCLC, NF-κB was first identified to regulate the expression of miR-21 by binding to its elements in the miR-21 gene promoter. We also found that NF-κB-induced miR-21 expression modulates its target gene, PTEN, the reduced expression of which promotes cell survival. Overexpression of PTEN in A549 cells could reverse the effect of miR-21 and NF-κB to restore the sensitivity of A549 cells to cisplatin.

In conclusion, our observations indicated that the NF-κB/miR-21/PTEN pathway contributes to the resistance of NSCLC to cisplatin treatment.

Author Contributions
Conceived and designed the experiments: WG ZY. Performed the experiments: ZY SF YD. Analyzed the data: WY. Contributed reagents/materials/analysis tools: WY YT. Wrote the paper: ZY WG. Designed the study and handled the submission: WG. Did most of the experiments and prepared the manuscript: ZY.

References
1. Blanco R, Maestu I, de la Torre MG, Cassinello A, Nunez I. A review of the management of elderly patients with non-small-cell lung cancer. Ann Oncol. 2014.
2. Reck M, Popat S, Reimmut N, de Ruyscher D, Kerr KM, Peters S, et al. Metastatic non-small-cell lung cancer (NSCLC): ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2014; 25 Suppl 3: ii27–39. doi:10.1093/annonc/mdu199 PMID: 25115305
3. Cancer Genome Atlas Research N. Comprehensive genomic characterization of squamous cell lung cancers. Nature. 2012; 489: 519–525. doi:10.1038/nature11404 PMID: 22960745
4. Cancer Genome Atlas Research N. Comprehensive molecular profiling of lung adenocarcinoma. Nature. 2014; 511: 543–550. doi:10.1038/nature13985 PMID: 25079552
5. Zhao N, Wilkerson MD, Shah U, Yin X, Wang A, Hayward MC, et al. Alterations of LKB1 and KRAS and risk of brain metastasis: Comprehensive characterization by mutation analysis, copy number, and gene expression in non-small-cell lung carcinoma. Lung Cancer. 2014; 86: 255–261. doi:10.1016/j.lungcan.2014.08.015 PMID: 25224251
6. Qiu Z, Dai Y. Roadmap of miR-122-related clinical application from bench to bedside. Expert Opin Investig Drugs. 2014; 23: 347–355. doi:10.1517/13543784.2014.867327 PMID: 24354366
7. Li Y, Liu Y, Wang Z, Hou H, Lin Y, Jiang Y. MicroRNA: Not Far from Clinical Application in Ischemic Stroke. ISRN Stroke. 2013; 2013: 1–7. PMID: 25285308

8. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology. 2007; 133: 647–658. PMID: 17681183

9. Meng F, Henson R, Lang M, Wehbe H, Maheshwari S, Mendell JT, et al. Involvement of human microRNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology. 2006; 130: 2113–2129. PMID: 16762633

10. Xu LF, Wu ZP, Chen Y, Zhu QS, Hamidi S, Navab R, MicroRNA-21 (miR-21) Regulates Cellular Proliferation, Invasion, Migration, and Apoptosis by Targeting PTEN, RECK and Bcl-2 in Lung Squamous Carcinoma, Gejiu City, China. PLoS One. 2014; 9: e103698. doi: 10.1371/journal.pone.0103698 PMID: 25084400

11. Umemura S, Mimaki S, Makinoshima H, Tada S, Ishii G, Ohmatsu H, et al. Therapeutic Priority of the PI3K/AKT/mTOR Pathway in Small Cell Lung Cancers as Revealed by a Comprehensive Genomic Analysis. J Thorac Oncol. 2014; 9: 1324–1331. doi: 10.1097/JTO.0000000000000250 PMID: 25122428

12. Shen H, Zhu F, Liu J, Xu T, Pei D, Wang R, et al. Alteration in Mir-21/PTEN Expression Modulates Gefitinib Resistance in Non-Small Cell Lung Cancer. PLoS One. 2014; 9: e103305. doi: 10.1371/journal.pone.0103305 PMID: 25058005

13. Niu J, Shi Y, Tan G, Yang CH, Fan M, Pfeffer LM, et al. DNA damage induces NF-kappaB-dependent microRNA-21 up-regulation and promotes breast cancer cell invasion. J Biol Chem. 2012; 287: 21783–21795. doi: 10.1074/jbc.M112.355495 PMID: 22547075

14. Qian WF, Guan WX, Gao Y, Tan JF, Qiao ZM, Huang H, et al. Inhibition of STAT3 by RNA interference suppresses angiogenesis in colorectal carcinoma. Brazilian Journal of Medical and Biological Research. 2011; 44: 1222–1230. PMID: 22030865

15. Xie B, Wang C, Zheng Z, Song B, Ma C, Thiel G, et al. Egr-1 transactivates Bim gene expression to promote neuronal apoptosis. J Neurosci. 2011; 31: 5032–5044. doi: 10.1523/JNEUROSCI.5504-10.2011 PMID: 21451041

16. Liu M, Ma S, Liu M, Hou Y, Liang B, Su X, et al. Synergistic killing of lung cancer cells by cisplatin and radiation via autophagy and apoptosis. Oncol Lett. 2014; 7: 1903–1910. doi: 24932256

17. Markou A, Tsaroucha EG, Kaklamanis L, Fotinou M, Georgoulias V, Lianidou ES. Prognostic value of mature microRNA-21 and microRNA-205 overexpression in non-small cell lung cancer by quantitative real-time RT-PCR. Clin Chem. 2008; 54: 1696–1704. doi: 10.1373/clinchem.2007.101741 PMID: 18719201

18. Seike M, Goto A, Okano T, Bowman ED, Schetter AJ, Horikawa I, et al. MiR-21 is an EGFR-regulated anti-apoptotic factor in lung cancer in never-smokers. Proc Natl Acad Sci U S A. 2009; 106: 12085–12090. doi: 10.1073/pnas.0905234106 PMID: 19997153

19. Zhu W, Xu B. MicroRNA-21 Identified as Predictor of Cancer Outcome: A Meta-Analysis. PLoS One. 2014; 9: e103373. doi: 10.1371/journal.pone.0103373 PMID: 25098165

20. Ma XL, Liu L, Liu XX, Li Y, Deng L, Xiao ZL, et al. Prognostic role of microRNA-21 in non-small cell lung cancer: a meta-analysis. Asian Pac J Cancer Prev. 2012; 13: 2329–2334. PMID: 22901216

21. Liu ZL, Wang H, Liu J, Wang ZX. MicroRNA-21 (miR-21) expression promotes growth, metastasis, and chemo- or radioresistance in non-small cell lung cancer cells by targeting PTEN. Mol Cell Biochem. 2013; 372: 35–45. doi: 10.1007/s11010-012-1443-3 PMID: 22956424
26. Zhang JG, Wang JJ, Zhao F, Liu Q, Jiang K, Yang GH. MicroRNA-21 (miR-21) represses tumor suppressor PTEN and promotes growth and invasion in non-small cell lung cancer (NSCLC). Clin Chim Acta. 2010; 411: 846–852. doi:10.1016/j.cca.2010.02.074 PMID: 20223231

27. Yang CH, Yue J, Fan M, Pfeffer LM. IFN induces miR-21 through a signal transducer and activator of transcription 3-dependent pathway as a suppressive negative feedback on IFN-induced apoptosis. Cancer Res. 2010; 70: 8108–8116. doi:10.1158/0008-5472.CAN-10-2579 PMID: 20813833

28. Li X, Gao L, Cui Q, Gary BD, Dyess DL, Taylor W, et al. Sulindac inhibits tumor cell invasion by suppressing NF-kappaB-mediated transcription of microRNAs. Oncogene. 2012; 31: 4979–4986. doi:10.1038/onc.2011.655 PMID: 22286762