MicroRNA-21 Regulates Non-Small Cell Lung Cancer Cell Invasion and Chemo-Sensitivity through SMAD7

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Key Words
Non-small cell lung cancer (NSCLC) • Carboplatin • SMAD7 • miR-21 • TGFβ receptor signaling • Cancer invasion

Abstract
Background/Aims: SMAD7 is a key inhibitor of transforming growth factor β (TGFβ) receptor signaling, which regulates the alteration of cancer cell invasiveness through epithelial-mesenchymal cell conversion. Carboplatin is a commonly used drug in the chemotherapy for non-small cell lung cancer (NSCLC). Nevertheless, the molecular mechanisms underlying its suppressive effects on the NSCLC cell invasion are not completely understood. In the current study, we addressed this question by analyzing the effects of Carboplatin on microRNA-regulated SMAD7. Methods: We used Carboplatin to treat NSCLC cell lines. We performed bioinformatics analyses on the binding of microRNA-21 (miR-21) to the 3'-UTR of SMAD7 mRNA, and verified the biological effects of this binding using promoter luciferase reporter assay. The effects of Carboplatin or miR-21-modification on NSCLC cell invasion were evaluated in either a transwell cell invasion assay, or a scratch wound healing assay. Results: We found that Carboplatin inhibited the NSCLC cell invasion, in either a transwell cell invasion assay, or a scratch wound healing assay. Moreover, Carboplatin increased the levels of SMAD7 protein, but not mRNA, in NSCLC cells, suggesting presence of post-transcriptional control of SMAD7 by Carboplatin. Furthermore, expression of miR-21 was found to be inhibited by Carboplatin, and bioinformatics analyses showed that miR-21 targeted the 3’-UTR of SMAD7 mRNA to inhibit its translation, which was confirmed by luciferase reporter assay. Conclusion: Carboplatin may upregulate SMAD7 through suppression of miR-21 to inhibit TGFβ receptor signaling mediated NSCLC cell invasion.

L. Lin and H.-b. Tu contributed equally.
Introduction

Non-small cell lung cancer (NSCLC) is a prevalent lung cancer of high incidence. NSCLC has been catalogued into three subtypes: squamous cell carcinoma, large cell carcinoma, and adenocarcinoma [1-4]. Some NSCLCs are resistant to chemotherapy and radiation therapy, and are fast growing and highly invasive [1-5]. Thus, great efforts have been made to elucidate the mechanisms underlying the invasion and chemo-sensitivity of NSCLC [3, 4, 6-9].

Cisplatin is a commonly used chemotherapeutic drug in the treatment of SCLC [10]. Due to Cisplatin’s non-hematologic toxicities, Carboplatin was developed and has far fewer non-hematologic toxicities [10]. Carboplatin has been shown to be effective as, but less toxic than, Cisplatin, in both SCLC and NSCLC. Moreover, Carboplatin has been combined with several different chemotherapeutic agents, including ifosfamide and paclitaxel in hopes of improving the responses and overall survival of the patients [10]. Although Carboplatin has been extensively used and studied in NSCLC treatment, the molecular mechanisms underlying its suppressive effects on the NSCLC cell invasion are not completely understood.

The epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells that can differentiate into a variety of cell types [11-13]. EMT is essential for numerous developmental processes including mesoderm formation and neural tube formation. EMT has also been shown to occur in wound healing, in organ fibrosis and in the initiation of cancer invasion [11-13]. Among all factors that induce EMT, transforming growth factor β1 (TGFβ1) has been shown to be the most potential one [11-13]. TGFβ receptor signaling pathway plays essential roles in many biological events [14-19]. When a ligand binds to a type II TGFβ receptor, it catalyzes the phosphorylation of a type I TGFβ receptor, which triggers phosphorylation of two intracellular proteins SMAD2 and SMAD3 to form heteromeric complexes with SMAD4. The activated SMAD complexes then translocate to the nucleus, where they regulate the transcription of target genes [14]. SMAD7 is a general antagonist against all superfamily signaling. SMAD7 can be induced at the transcriptional level [20], predominantly after binding of superfamily ligands to a receptor [21, 22]. SMAD7 can block R-SMAD phosphorylation [23], degrade type I receptors [24], and even exert an inhibitory effect in the nucleus [25]. Although TGFβ receptor signaling pathway has been shown to play critical roles during EMT in many cancers, its involvement in the NSCLC chemo-sensitivity upon Carboplatin treatment is ill-defined.

MicroRNA (miRNA) is a class of non-coding small RNAs consisting of 18-23 nucleotides. MiRNAs regulate the protein translation via its base-pairing with the 3′-untranslated region (3′-UTR) of the mRNA of the target genes to affect cell proliferation, apoptosis and differentiation [26-28]. MiRNAs have been well defined as a regular for tumorigenesis [29-35]. Among all miRNAs, miR-21 was recently reported as an aberrantly expressed miRNA in NSCLC [36-40], but the alteration of miR-21 during the Carboplatin treatment is not well defined.

Here, we examined the effects of Carboplatin on NSCLC cells and the underlying mechanisms.

Materials and Methods

Experimental protocol approval

All experimental protocols were approved by the Research Bureau of Shanghai Pulmonary Hospital. All mouse experiments were approved by the Institutional Animal Care and Use Committee at Shanghai Pulmonary Hospital (Animal Welfare Assurance). The methods regarding animals were carried out in "accordance" with the approved guidelines.

NSCLC cell lines and reagents

Two human NSCLC lines A549 (origin from carcinoma) and NCI-H23 (H23, origin from non-small cell lung cancer) were both purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). These
two cell lines were used in the current study, since they represent different types of lung cancer. A549 cell line was first developed in 1972 by Dr. Giard through the removal and culturing of cancerous lung tissue in the explanted tumor from a 58-year-old Caucasian male [41]. H23 cell line was developed in 1980 by Dr. Gazdar from a 51-year-old black male [42]. The two cell lines were all cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin and streptomycin in a 5% CO₂ humidified cell-culture incubator at 37°C. Carboplatin (Sigma-Aldrich) was prepared in a stock of 1mmol/l and applied to the cultured NSCLC cells at 5µmol/l.

**Plasmids and cell transfection**

MiR-21-modulating and SMAD7-modulating plasmids were prepared using a backbone plasmid containing a GFP reporter under CMV promoter (pcDNA3.1-CMV-GFP, Clontech, Mountain View, CA, USA). The miR-21 mimic, or antisense, or control null, or short-hairpin interfering RNA for SMAD7 (shSMAD7), or a control scrambled sequence was all purchased from Sigma-Aldrich, and digested with Xhol and BamHI and subcloned with a 2A into a pcDNA3.1-CMV-GFP backbone. The small 2A peptide sequences, when cloned between genes, allow for efficient, stoichiometric production of discrete protein products within a single vector through a novel "cleavage" event within the 2A peptide sequence. Sequencing was performed to confirm the correct orientation of the new plasmid. Transfection was performed with Lipofectamine 2000 reagent (Invitrogen), according to the instructions of the manufacturer. One day after transfection, the transfected cells were purified by flow cytometry based on their expression of GFP.

**In vivo implantation of tumor cells and quantification of tumor size**

MiR-21-modified and control A549 cells (10⁶; miR-21/null) were subcutaneously injected under the skin at the back of the NOD/SCID mice. The tumor was allowed to grow for 1 month, dissected out, and then weighed for quantification.

**MicroRNA target prediction and 3'-UTR luciferase-reporter assay**

MiRNAs targets were predicted with the algorithms TargetSan (https://www.targetscan.org) [43]. The SMAD7 3'-UTR reporter plasmid (pRL-SMAD7 3'-UTR) and SMAD7 3'-UTR reporter plasmid with a mutant at the miR-21 binding site (pRL-SMAD7 3'-UTR mut) were purchased from Creative Biogene (Shirley, NY, USA). NSCLC cells were co-transfected with pRL-SMAD7 3'-UTR/pRL-SMAD7 3'-UTR mut and miR-21/amiR-21/null by Lipofectamine 2000 (5×10⁴ cells per well). Cells were collected 24 hours after transfection for assay using the dual-luciferase reporter assay system gene assay kit (Promega, Beijing, China), according to the manufacturer’s instructions.

**Western blot**

Protein was extracted from the cultured cells with RIPA lysis buffer (1% NP40, 0.1% Sodium dodecyl sulfate (SDS), 100µg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12000×g at 4°C for 20min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4×SDS loading buffer (125mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100mmol/l Dithiothreitol (DTT), and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated secondary antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies were anti-SMAD7, anti-MMP2, anti-MMP9, anti-ZEB1, anti-Snail1 and anti-α-tubulin (Cell Signaling, San Jose, CA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). The protein levels were first normalized to α-tubulin, and then normalized to the experimental controls.

**Quantitative real-time PCR (RT-qPCR)**

Total RNA was extracted from cultured cells using miRNeasy kit (Qiagen), for cDNA synthesis. Quantitative real-time NSCLCR (RT-qPCR) was performed in duplicates with QuantiTect SYBR Green NSCLCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed with 2⁻ΔΔCt method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to experimental controls.
Transwell cell invasion assay
Cells (10^4) were plated into the top side of polycarbonate transwell filter coated with Matrigel in the upper chamber of the BioCoatTM Invasion Chambers (Becton-Dickinson Biosciences, Bedford, MA, USA) and incubated at 37°C for 22 hours. The cells inside the upper chamber with cotton swabs were then removed. Migratory and invasive cells on the lower membrane surface were fixed, stained with hematoxylin, and counted for 10 random 100X fields per well. Cell counts are expressed as the mean number of cells per field of view. Five independent experiments were performed and the data are presented as mean ± standard deviation (SD).

Scratch wound healing assay
Scratch wound healing assay was performed as has been described previously [44]. Cells were seeded in 24-well plates at a density of 104 cells/well in complete media and cultured to confluence. The cell monolayer was serum starved overnight in media prior to initiating of the experiment. Confluent cell monolayer were then scraped with a yellow pipette tip to generate scratch wounds and washed twice with media to remove cell debris. Cells were incubated at 37 °C for 24 hours. Time lapse images were captured after 12 hours. Images were captured from 5 randomly selected fields in each sample, and the migration areas are determined by subtracting the wound area at the indicated time periods from the initial wound area, using by NIH ImageJ (Bethesda, MA, USA), as has been previously described [45].

Statistical analysis
All statistical analyses were carried out using the GraphPad Prism 6.0 statistical software package (GraphPad Software, Inc. La Jolla, CA, USA). All values are depicted as mean ± standard deviation (SD) and are considered significant if p < 0.05. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher’ Exact Test for comparison of two groups.

Results
Carboplatin inhibits NSCLC cell invasion
We examined the effect of Carboplatin on NSCLC cell invasion. We gave Carboplatin at 5µmol/l to 2 cultured human NSCLC cell lines, A549 and H23. We found that Carboplatin significantly decreased the cell invasion in either a scratch wound healing assay (Fig. 1A), or a transwell cell invasion assay (Fig. 1B-C) in A549 cells. Moreover, Carboplatin significantly decreased the cell invasion in either a scratch wound healing assay (Fig. 1D), or a transwell cell invasion assay (Fig. 1E-F) in H23 cells. These data thus suggest that Carboplatin may suppress NSCLC cell growth invasion.

Carboplatin increases SMAD7 and decreases miR-21 in NSCLC cells
Since TGFβ receptor signaling plays a pivotal role in the EMT and cancer invasion in NSCLC cells, we examined the effect of Carboplatin on the expression of key proteins of TGFβ receptor signaling. Specifically, we found that Carboplatin significantly increased the protein levels of SMAD7, the pan- TGFβ receptor signaling inhibitor, in both A549 cells and H23 cells (Fig. 2A). However, the mRNA levels of SMAD7 were not affected by Carboplatin (Fig. 2B). These data suggest that Carboplatin may regulate the translation of SMAD7 at post-transcriptional level. Since miRNAs are key regulator for protein translation, we thus screened SMAD7-targetting miRNAs using bioinformatics analyses. We found that miR-21 was such a SMAD7-targetting miRNA (Fig. 2C) and the expression of miR-21 was significantly reduced by Carboplatin treatment (Fig. 2D).

MiR-21 targets 3′-UTR of SMAD7 mRNA to inhibit its protein translation in NSCLC cells
In order to examine whether the binding of miR-21 to SMAD7 mRNA may affect SMAD7 protein translation in NSCLC cells, we either overexpressed miR-21, or inhibited miR-21 in the A549 cells, through transfecting the cells with a miR-21-expressing plasmid (miR-21), or with a plasmid carrying miR-21 antisense (as-miR-21). The A549 cells were also
transfected with a null plasmid as a control (null). The modification of miR-21 levels in A549 cells was confirmed by RT-qPCR (Fig. 3A). MiR-21-modified A549 cells were then transfected with 1μg plasmids carrying luciferase reporter for 3'-UTR of SMAD7 mRNA. Moreover, null-transfected A549 cells were also transfected with 1μg plasmids carrying luciferase reporter
for 3'-UTR of SMAD7 mRNA with one mutate at the miR-21 binding site (mut). The luciferase activities were quantified in these cells, suggesting that miR-21 specifically targets 3'-UTR of SMAD7 mRNA to inhibit its translation (Fig. 3B).
MiR-21 promotes NSCLC cell invasion through suppressing SMAD7

We found that overexpression of miR-21 in A549 cells increased in cell invasion in a scratch wound healing assay (A), and in a transwell cell invasion assay (B). On the other hand, depletion of miR-21 in A549 cells decreased cell invasion, in a scratch wound healing assay (A), and in a transwell cell invasion assay (B). (C-D) Overexpression of miR-21 in H23 cells increased in cell invasion in a scratch wound healing assay (C), and in a transwell cell invasion assay (D). On the other hand, depletion of miR-21 in H23 cells decreased cell invasion, in a scratch wound healing assay (C), and in a transwell cell invasion assay (D). (E) Western blot for MMP2, MMP9, ZEB1 and Snail1. *p<0.05. N=5.

MiR-21 promotes NSCLC cell invasion through suppressing SMAD7

We found that overexpression of miR-21 in A549 cells increased in cell invasion in a scratch wound healing assay (Fig. 4A), and in a transwell cell invasion assay (Fig. 4B). On the other hand, depletion of miR-21 in A549 cells decreased cell invasion, in a scratch wound healing assay (Fig. 4A), and in a transwell cell invasion assay (Fig. 4B). Suppression of SMAD7 abolished the effects of miR-21 depletion on cell invasion (Fig. 4A-B). Similarly, we found that overexpression of miR-21 in H23 cells increased in cell invasion in a scratch wound healing assay (Fig. 4C), and in a transwell cell invasion assay (Fig. 4D). On the other hand, depletion of miR-21 in H23 cells decreased cell invasion, in a scratch wound healing assay (Fig. 4C), and in a transwell cell invasion assay (Fig. 4D). Suppression of SMAD7 abolished the effects of miR-21 depletion on cell invasion (Fig. 4C-D). In addition, we analyzed the effects of miR-21 modification on metastasis-associated proteins, and found that MMP2, MMP9, ZEB1 and Snail1 may be downstream targets of miR-21/SMAD7 (Fig. 4E).
MiR-21 increases NSCLC cell growth in vivo

Finally, miR-21-modified A549 cells (miR-21) or control cells (null) were implanted into NOD/SCID mice at $10^6$ cells per mice to examine the effects of miR-21 levels on tumor growth. One month after tumor cell transplantation, the tumor was dissected out and weighed. The weight of miR-21-overexpressing A549-cell-formed tumor was significantly higher than control, shown by gross images (A), and by quantification (B). *p<0.05. N=5.

**Fig 5.** MiR-21 increases NSCLC cell growth in vivo. MiR-21-modified A549 cells (miR-21) or control cells (null) were implanted into NOD/SCID mice at $10^6$ cells per mice to examine the effects of miR-21 levels on tumor growth. One month after tumor cell transplantation, the tumor was dissected out and weighed. The weight of miR-21-overexpressing A549-cell-formed tumor was significantly higher than control, shown by gross images (A), and by quantification (B). *p<0.05. N=5.

**Discussion**

In the current study, we analyzed the effects of Carboplatin on the cell invasion of NSCLC cells. We found that Carboplatin significantly inhibited NSCLC cell invasion, in two assay independently. This result confirmed the previous findings showing that Carboplatin is an effective therapeutic treatment for NSCLC.

SMAD7 has been shown to regulate cancer cell metastases in some types of cancer [46]. MiR-21 has been shown to regulate NSCLC through WNT signaling [47], or PTEN [39]. However, a direct regulatory relationship between SMAD7 and miR-21 has never been acknowledged. Since we identified SMAD7, as a target of Carboplatin on the control of the NSCLC cell invasion, and since the regulation of SMAD7 by Carboplatin appeared to be at protein levels, rather than at mRNA levels, these data suggest that the regulation of SMAD7 by Carboplatin may be through gene transcription, but through protein translation.
or degradation. The modulation of SMAD7 protein could be either through alteration in protein translation by miRNAs, or through alteration in protein degradation via protein phosphorylation/de-phosphorylation, or sumoylation/de-sumoylation, or acetylation/de-acetylation, or ubiquitination, whereas the latter did not appear to play a role in SMAD7 regulation.

MiR-21 is a miRNA that has been extensively studied. Most importantly, Zhang et al. reported that miR-21 was overexpressed in NSCLC tumor tissues relative to adjacent non-tumor tissues. Notably, patients with advanced clinical TNM stage or distal metastasis showed higher miR-21 expression than those without them. They have identified an inverse correlation between miR-21 and PTEN protein in tumor tissue and further showed that miR-21 post-transcriptionally down-regulates the expression of tumor suppressor PTEN and stimulates growth in NSCLC cells [40]. Later on, the regulatory axis of miR-21 and PTEN in NSCLC has been confirmed by follow-up studies [38, 39, 48-50]. Nevertheless, the regulation of SMAD7, and subsequently TGFβ receptor signaling, has not been reported before.

We have tried carboplatin on miR-21-depleted A549 cells and the effects appeared to be attenuated, and the remaining effects probably have resulted from other mechanisms of carboplatin, as has been studied before [10].

Here, we provided compelling data to demonstrate that miR-21 also targets SMAD7 to activate EMT-associated NSCLC cell invasion, and Carboplatin significantly decreased miR-21 levels to enhance protein translation of SMAD7. Thus, the alteration of miR-21 levels appeared to be one marker for the chemo-sensitivity for Carboplatin, and may be used as a predictor for the prognosis of the NSCLC patients after Carboplatin treatment. Future approaches may address this question in clinic. Although here we identified miR-21 as a direct regulator of SMAD7 protein translation, we do not exclude the possibility of modification of SMAD7 protein levels through miRNAs other than miR-21, which may be examined in future studies. On the other hand, as discussed above, miR-21 also targeted PTEN to regulate NSCLC cell growth. These data reflect the complexity of the regulation of tumorigenesis by a network of molecules in NSCLC.

Of note, here we used 2 NSCLC cell lines, which may substantially exclude a possibility of cell-line specific results in the current study. Together, our data shed light on a previously unrecognized signaling regulatory mechanisms that underlies the effects of Carboplatin against NSCLC cells. Carboplatin may suppress miR-21 levels in NSCLC cells, which subsequently upregulates SMAD7 to suppress the NSCLC cell invasion.

Disclosure Statement

The authors have declared that no competing interests exist.

Reference


