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Reduced miR-363-3p expression in non-small cell lung cancer is associated with gemcitabine resistance via targeting of CUL4A

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Abstract. – OBJECTIVE: Accumulating evidence has suggested that aberrant expression of microRNAs (miRNAs) is associated with non-small cell lung cancer (NSCLC) proliferation, migration, invasion and chemotherapy resistance. Cullin4A (CUL4A) has been previously reported to desensitize NSCLC cells to chemotherapy treatment. However, whether miRNAs regulate CUL4A to promote chemotherapy resistance remains unknown.

PATIENTS AND METHODS: Tissues were obtained from 40 NSCLC patients who received surgery at the Yancheng City No. 1 People's Hospital. Cell Counting Kit-8 (CCK-8) assays were applied for the detection of cell proliferation; mRNA and protein levels were determined by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) and Western blot, respectively. The interaction between mRNA 3'UTR and miRNA was predicted by TargetScan and verified by Dual-Luciferase reporter assay.

RESULTS: In the present study, miR-363-3p levels were revealed to be significantly decreased in tumor tissues obtained from NSCLC patients compared with adjacent normal tissues. The results of the CCK-8 assays showed that the overexpression of miR-363-3p may slightly inhibit the proliferation of A549 and H23 cells. Notably, the transfection with miR-363-3p antagonists reduced the sensitivity of A549 and H23 cells to gemcitabine treatment, whereas the overexpression of miR-363-3p markedly increased the sensitivity of A549 and H23 cells to gemcitabine treatment. Furthermore, CUL4A mRNA and protein levels were revealed to be decreased in A549 cells transfected with miR-363-3p mimics. The Dual-Luciferase reporter assay results further suggested that CUL4A represents a target gene of miR-363-3p.

CONCLUSIONS: The results indicated that decreased miR-363-3p expression enhanced gemcitabine resistance in NSCLC cells *via* regulation of CUL4A.

Key Words:
MiR-363-3p, CUL4A, Gemcitabine, Non-small cell lung cancer.

Introduction

Lung cancer is the leading cause of death in both males and females[1]. Non-small cell lung cancer (NSCLC) accounts for more than 80% of lung cancer cases, and it has a highly aggressive nature[2].

Gemcitabine-based chemotherapy is the first-line therapeutic approach for the treatment of NSCLC patients[3]. However, due to acquired chemotherapy resistance, patients with NSCLC undergoing chemotherapy treatment may exhibit little improvement, and the prognosis of NSCLC patients receiving gemcitabine treatment remains poor[4-6]. Therefore, the molecular mechanism underlying chemotherapy resistance requires further investigation to improve the clinical outcomes of NSCLC patients.

MicroRNAs (miRNAs) are small, non-coding, single-stranded RNAs that suppress target gene expression by binding to the 3'UTR of mRNAs[7]. Via regulation of different target genes, a miRNA may exhibit an oncogene role or a tumor suppressor role according to different cell types[8]. Increasing evidence has suggested that miRNAs may also modulate chemotherapy sensitivity in cancer cells; however, the underlying molecular mechanism remains largely unknown[9].

The downregulation of miR-363-3p has been observed in various cancer types, including thyroid carcinoma, lung adenocarcinoma and ovarian cancer, and has been demonstrated to inhibit cancer progression[10-12]. Interestingly, de-

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creased miR-363-3p levels have also been revealed to be associated with chemotherapy resistance in numerous cancer types, including hepatocellular carcinoma, leukemia and breast cancer[13-15]. However, the role of miR-363-3p in chemotherapy resistance of NSCLC remains largely unknown.

As a member of the cullin protein family, Cullin4A (CUL4A) is a ubiquitin ligase protein that is associated with DNA replication, cell cycle regulation and genomic instability[16-18]. The overexpression of CUL4A has been previously reported[19-22] in numerous cancer types, including NSCLC. In NSCLC, CUL4A forms a complex with FBXW5 to facilitate DLC1 degradation and promote cancer cell growth[23]. A previous research[24] has shown that silencing of CUL4A expression increases the sensitivity of NSCLC cells to gemcitabine treatment. However, the regulation of CUL4A by miRNA in NSCLC has not been investigated yet.

In the current work, miR-363-3p expression levels in tumor tissues and adjacent normal tissues obtained from NSCLC patients, as well as the function and mechanism of miR-363-3p in the regulation of chemotherapy sensitivity in NSCLC cells, were investigated. The results of the present work demonstrated that tumor tissues exhibited decreased levels of miR-363-3p. In addition, the overexpression of miR-363-3p was revealed to slightly inhibit cell proliferation of NSCLC cells. However, the results demonstrated that the overexpression of miR-363-3p greatly enhanced gemcitabine-induced cell growth arrest, and transfection with miR-363-3p antagonists was revealed to desensitize cells to gemcitabine treatment. Furthermore, the present work predicted and validated CUL4A as a target gene of miR-363-3p. These data suggested that miR-363-3p has a pivotal role in mediating the sensitivity of NSCLC cells to chemotherapy treatment.

Materials and Methods

Human NSCLC Tissues and Normal Tissues

A total of 40 tumor tissues and matched adjacent normal tissues were collected from NSCLC patients in Yancheng City No. 1 People's Hospital between the 15th May 2014 and 30th October 2016. Specimens were surgically removed and immediately stored at -80˚C prior to further experiments. None of the patients had received preoperative radiotherapy or chemotherapy before enrolling in the present study. The work was carried out under the supervision of the Ethics Committee of Yancheng City No. 1 People's Hospital. The informed consent was obtained from all participants.

Cell Culture and Reagents

Normal human lung epithelial cell line BEAS-2B, as well as A549 and H23 human NSCLC cell lines, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and used within 6 months post-collection. BEAS-2B, A549 and H23 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA) and 1% penicillin-streptomycin solution (Gibco, Grand Island, NY, USA) were cultured at 37˚C in a humidified incubator containing 5% CO2. Gemcitabine was purchased from MedChem Express (Monmouth Junction, NJ, USA).

Cell Viability Assay

The cell growth rate was measured using a Cell Counting Kit-8 assay (CCK-8, Dojindo, Kumamoto, Japan). Briefly, cells were cultured in 96-well plates for 0, 24, 48 and 72 h time intervals. A total of 10 μL CCK-8 solution was subsequently added into the indicated wells and further incubated for 2 h. Following this, the solution containing CCK-8 was transferred into another 96-well plate, and the absorbance at 450 nm was detected using a microplate reader (Bio-Rad, Hercules, CA, USA). To determine the sensitivity of cells to treatment with gemcitabine, the cells were treated with increasing concentrations of gemcitabine (5, 50, 500 and 5000 nM) for 48 h, and the cell viabilities were subsequently determined using a CCK-8 assay.

RNA Extraction and Real Time Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using gene-specific primers or random hexamers with the SuperScript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). Following this, a SYBR Premix Ex Taq kit (TaKaRa, Otsu, Shiga, Japan) and a Mir-X™ miRNA qRT-PCR SYBR Kit (TaKaRa, Otsu, Shiga, Japan) were

used for reverse transcription of mRNA and miRNA, respectively. GAPDH and U6 served as internal controls for mRNA and miRNA qPCR analyses, respectively. The thermo cycle condition was initial denaturation at 95°C for 30s followed by 40 cycles of denaturation at 95°C for 5s, annealing at 58°C for 15s, and elongation at 72°C for 10s. Stem-loop primers for miRNA qPCR were purchased from RiboBio (Guangzhou, China). The primers for CUL4A and GAPDH were synthesized by GeneScript (Nanjing, China). The primer sequences used for PCR were as follows: CUL4A forward, 5'-GTGGAAGATGGAGACAAGTTCA-3' and reverse, 5'-GTGTTTCATGAAGGGGAACCG-3'; GAPDH forward, 5'-AGCCACATCGCTCAGACA-3' and reverse, 5'-TGGACTCCACGACGTACT-3'; miR-363-3p forward, 5'-GCCGAGAATTGCACGGTAT-3' and reverse: 5'-CTCAACTGGTGTCGTGGA-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-ACGCTTCACGAATTTGCGT-3'. The expressions of mRNAs and miRNAs were calculated using 2-ΔΔCq method (25).

Western Blot

Lysates were prepared from cells using Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). After that, the concentration of protein lysates was analyzed using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 10 μg protein lysates were loaded into each lane of a 10% SDS gel and the proteins were separated by electrophoresis. Protein was subsequently transferred to a polyvinylidene difluoride (PVDF) membrane and then blocked using 5% non-fat milk for 1 h at 37°C. Following this, the membranes were incubated with indicated primary antibodies against CUL4A (#2699, 1:2000) and GAPDH (#97166, 1:5000) (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with Tris-Buffered Saline with Tween 20 (TBST) and subsequently incubated with anti-mouse (SA00001-1, 1:10000) and anti-rabbit secondary (SA00001-2, 1:10000) antibodies (ProteinTech, Chicago, IL, USA) for 1 h at 37˚C. An ECL substrate (Millipore, Billerica, MA, USA) was then used to determine protein expression. Protein bands were subsequently visualized using an Image Reader LAS-4000 (Fujifilm, Tokyo, Japan) and then analyzed using Image J software.

MiRNA Transfection

MiR-363-3p mimics, miR-NC mimics, miR-363-3p antagonists and miR-NC antagonists were purchased from GenePharma (Shanghai, China). MiRNA mimics and miRNA antagonists were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The sequences of miRNA mimics and miRNA antagonists were: miR-363-3p mimics: 5'- AAUUGCACGGUAUCCAUCUGUAUU-3'; miR-NC mimics: 5'-AUUGGAACGAUACAGAGAAGAUU-3'; miR-363-3p antagonist: 5'-UACAGAUGGAUACCGUGCAAUU-3'; miR-NC antagonist: 5'-UUCUCCGAACGUGUCACGUTT-3'.

Target Prediction and Dual-Luciferase Reporter Assay

Target genes of miR-363-3p were predicted using miRanda (http://www.microrna.org) and TargetScan (http://www.targetscan.org/). The predicted target genes were validated by performing Dual-Luciferase reporter assays. The 3'UTR of CUL4A mRNA was amplified from human genomic DNA and then cloned into pGL3 plasmids (Promega, Madison, WI, USA). Mutated CUL4A 3'UTRs were synthesized by introducing 2 site mutations into the pGL3-CUL4 3'UTR-WT. To perform the Dual-Luciferase reporter assay, A549 cells were co-transfected with reporter plasmids exhibiting either miR-363-3p mimics or miR-NC mimics. After incubation at 37°C for 48 h, the Firefly Luciferase activity and Renilla Luciferase activity of each well were detected using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7, and all data are presented as the mean±SD. The differences between the two groups were analyzed using the Student's t-test. The differences among the three groups were compared with one-way ANOVA, followed by Newman Keuls test. $p<0.05$ was considered statistically significant.

Results

Downregulation of MiR-363-3p in NSCLC

To investigate the expression levels of miR-363-3p in NSCLC, 40 paired tumor tissues and

normal tissues were obtained from NSCLC patients and RT-qPCR was subsequently performed. The results showed that miR-363-3p expression was significantly decreased in tumor tissues compared with normal tissues (Figure 1A), particularly in tumor tissues obtained from patients with advanced clinical stage NSCLC (Figure 1B). In addition, the results demonstrated that miR-363-3p levels were decreased in A549 and H23 cells compared with BEAS-2B cells (Figure 1C). Our RT-PCR results in NSCLC tumor tissues and cells indicated a tumor suppressor role of miR-363-3p.

MiR-363-3p Slightly Inhibited NSCLC Cell Growth

Cell proliferation assays were performed to analyze the effect of miR-363-3p overexpression on the growth of A549 cells. The transfection of miR-363-3p significantly enhanced miR-363-3p levels in A549 and H23 cells (Figure 2A). Furthermore, enhanced levels of miR-363-3p slightly inhibited A549 and H23 cell growth (Figure 2B-C).

MiR-363-3p Sensitized NSCLC Cells to Treatment with Gemcitabine

In addition, whether the miR-363-3p expression is associated with chemotherapy resistance exhibited by NSCLC cells was investigated. The miR-363-3p expression in A549 and H23 cells was suppressed via transfection with miR-363-3p antagonists (Figure 3A). Compared with A549 cells transfected with the miR-NC antagonist, the transfection with miR-363-3p antagonist partially attenuated decreased levels of cell viability post-treatment with gemcitabine, thus suggesting that miR-363-3p inhibition reduced the sensitivity of A549 cells to gemcitabine treatment (Figure 3B). Furthermore, the downregulation of miR-363-3p also desensitized H23 cells to gemcitabine treatment (Figure 3C). However, the overexpression of miR-363-3p markedly reduced the viability of A549 and H23 cells following gemcitabine treatment (Figure 3C-D). These results demonstrated that miR-363-3p may function as a sensitizer of NSCLC cells upon gemcitabine.

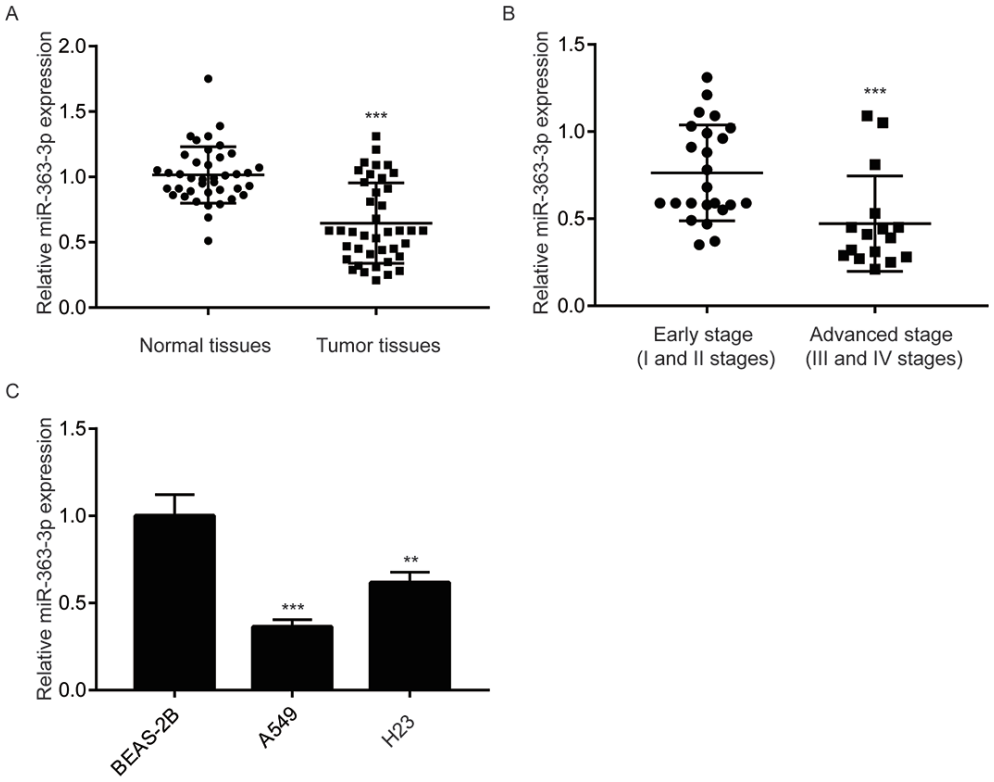

Figure 1. miR-363-3p was decreased in NSCLC tumor tissues and NSCLC cell lines. ***A,*** Compared with matched normal tissues, miR-363-3p levels were decreased in tumor tissues obtained from NSCLC patients. ***B,*** Compared with tumor tissues obtained from patients with early stage NSCLC (I and II phases), miR-363-3p levels were decreased in tumor tissues obtained from patients with advanced stage NSCLC (III and IV phases). ***C,*** Compared with normal lung epithelial cell line BESA-2B, miR-363-3p levels were decreased in NSCLC cell lines. $^{**}p<0.01$ and $^{***}p<0.001$.

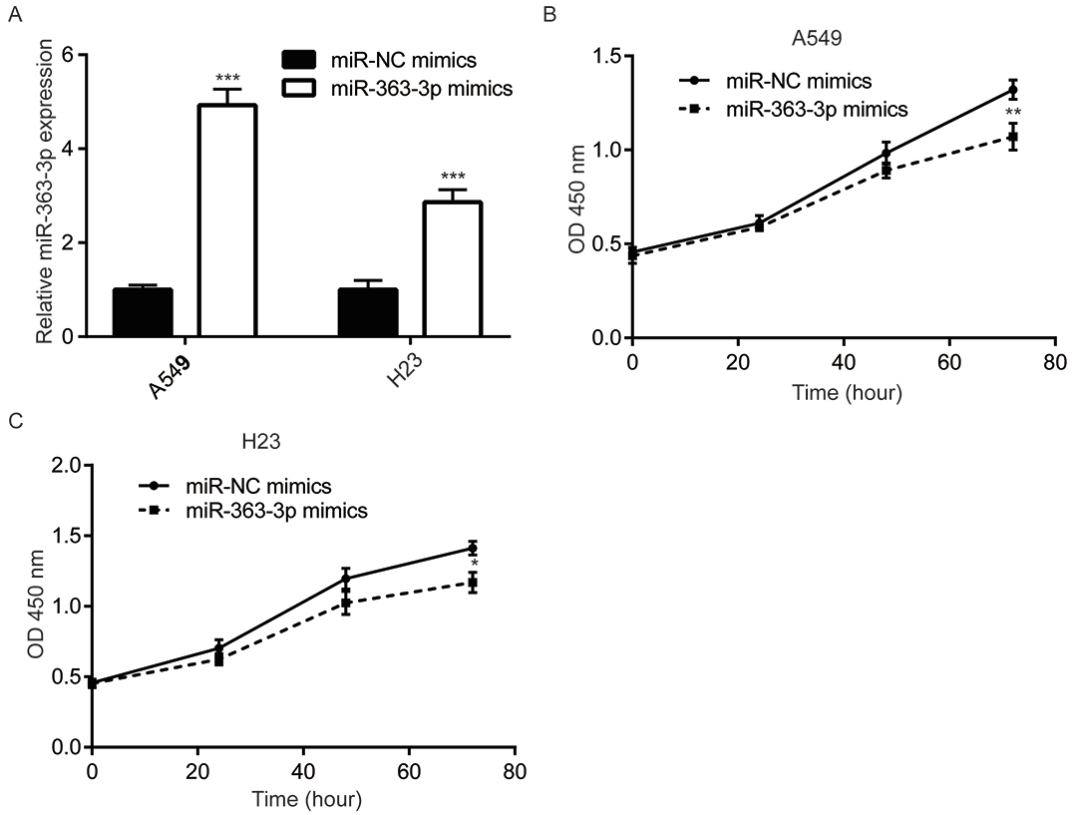

Figure 2. The overexpression of miR-363-3p inhibited the growth of NSCLC cells. ***A,*** Transfection with miR-363-3p mimics significantly enhanced miR-363-3p levels in both A549 and H23 cells. ***B-C,*** The overexpression of miR-363-3p slightly inhibited the growth of A549 and H23 cells. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

MiR-363-3p Negatively Regulated CUL4A in NSCLC Cells

Silencing of CUL4A was revealed to increase the sensitivity of NSCLC cells to chemotherapy[24]. RT-qPCR analyses demonstrated that the overexpression of miR-363-3p decreased CUL4A mRNA levels in A549 and H23 cells (Figure 4A). Additionally, the overexpression of miR-363-3p was revealed to reduce the protein levels of CUL4A (Figure 4B-C). Expression levels of TIEG1 and TGFB1 have been previously observed[24,27-28] to be associated with chemotherapy sensitivity and to be regulated by CUL4A in NSCLC cells. In both A549 and H23 cells, the overexpression of miR-363-3p was revealed to enhance TIEG1 and TGFB1 protein levels (Figure 4D-E). These results indicated that miR-363-3p may increase chemotherapy sensitivity via regulation of CUL4A.

CUL4A Was a Target Gene of MiR-363-3p in NSCLC Cells

To determine whether CUL4A is a target gene of miR-363-3p, bioinformatic analyses using TargetScan and miRanda were performed. The results of sequence alignment indicated that the CUL4A 3'UTR contained sequences that were complementary to miR-363-3p (Figure 5A). Furthermore, Dual-Luciferase reporter assays were performed to investigate the association between CUL4A and miR-363-3p. The results demonstrated that the overexpression of miR-363-3p decreased the relative Luciferase activity of A549 cells transfected with CUL4A 3'UTR-WT (Figure 5B). However, miR-363-3p mimics did not affect the relative Luciferase activity of A549 cells transfected with CUL4A 3'UTR-Mut (Figure 5C). These results suggested that miR-363-3p may bind to the 3'UTR of CUL4A to suppress its expression.

MiR-363-3p Sensitized NSCLC Cells to Gemcitabine Through Repression of CUL4A

To figure out whether CUL4A was pivotal for the regulation of gemcitabine sensitivity by miR-363-3p, we applied CUL4A siRNA to silence CU-

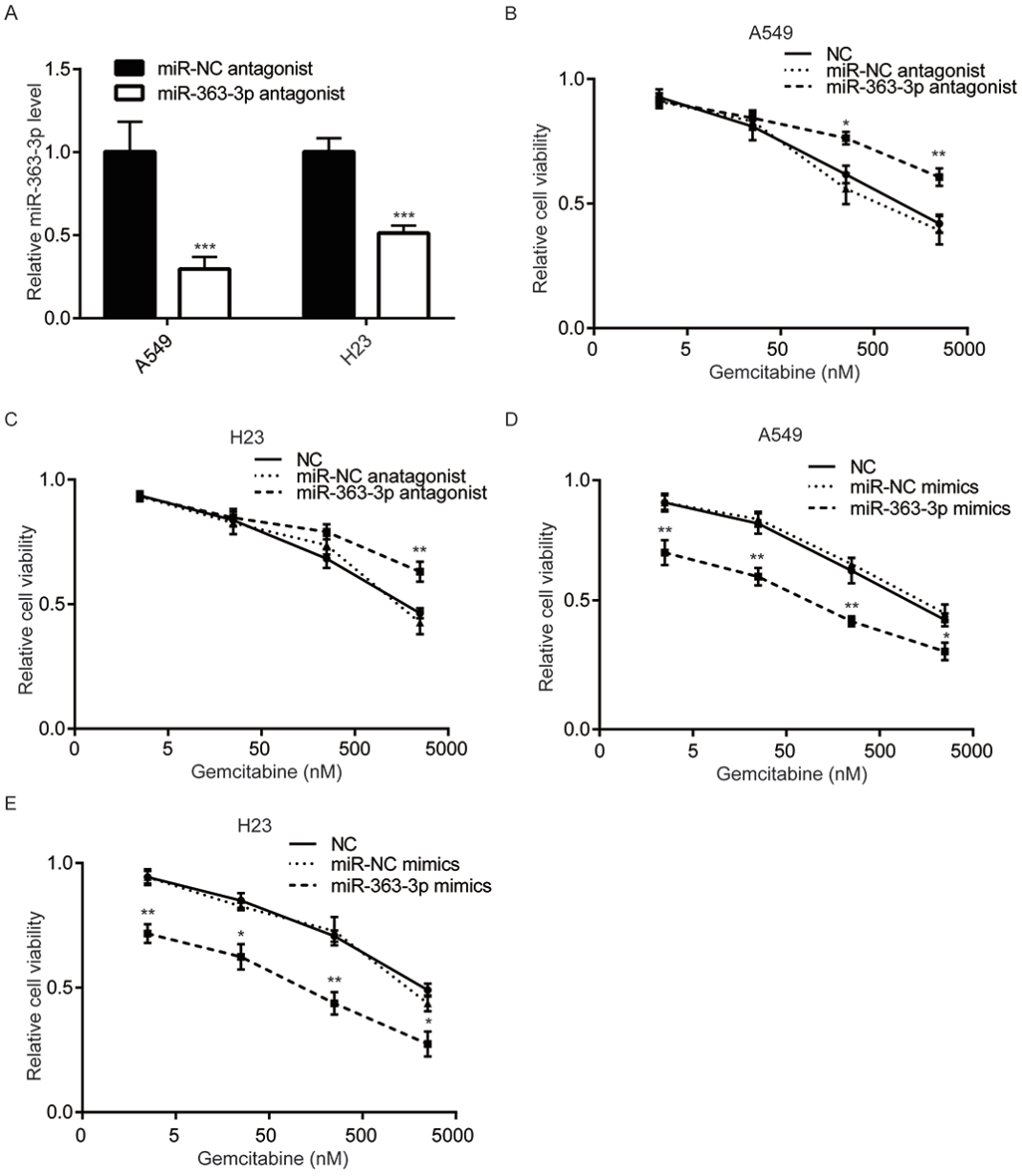

Figure 3. The miR-363-3p expression was associated with gemcitabine sensitivity in NSCLC cells. ***A,*** Transfection with miR-363-3p antagonist significantly decreased miR-363-3p levels in both A549 and H23 cells. ***B-C,*** The inhibition of miR-363-3p decreased the sensitivity of A549 and H23 cells to increasing concentrations of gemcitabine. ***D-E,*** Increased levels of miR-363-3p enhanced the sensitivity of A549 and H23 cells to gemcitabine treatment. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

L4A expression in NSCLC cells (Figure 6A-B). Compared with A549 and H23 cells transfected with miR-NC antagonist, miR-363-3p antagonist partially attenuated decreased levels of cell viability post-treatment with gemcitabine (Figure 6C-D). However, silencing of CUL4A reversed decreased gemcitabine sensitivity of NSCLC cells induced by miR-363-3p downregulation (Figure 6C-D).

CUL4A mRNA Levels Were Negatively Correlated With MiR-363-3p Levels in NSCLC Tumor Tissues

To investigate the expression association between CUL4A and miR-363-3p in NSCLC, RT-qPCR was performed to detect CUL4A mRNA levels in 40 tumor tissues collected from patients with NSCLC. The correlation analysis suggested

that the expression of CUL4A mRNA was negatively correlated with miR-363-3p levels in NSCLC tumor tissues (Figure 7).

Discussion

The efficacy of gemcitabine is often limited due to chemotherapy resistance exhibited by NSCLC patients[28]. Some authors[29,30] have demonstrated that aberrant expression of miRNAs is associated with chemotherapy resistance, and several miRNAs are involved in the development of chemotherapy resistance by targeting of specific mRNAs. In this work, the results revealed that miR-363-3p expression is associated with gemcitabine resistance in NSCLC cells.

It has been previously revealed[31] that numerous miRNAs functioning as tumor suppressors are downregulated in NSCLC. A previous

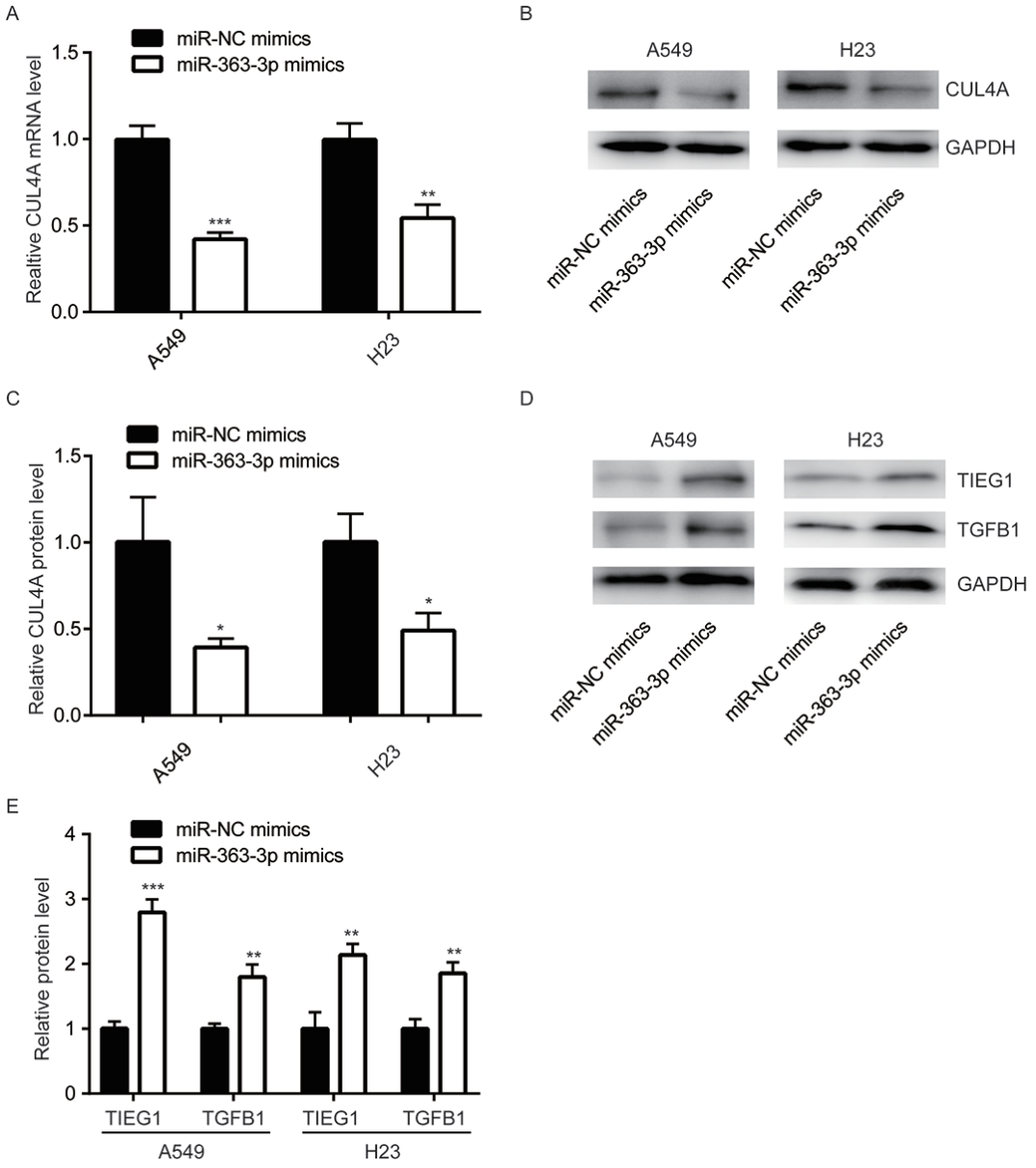

Figure 4. miR-363-3p overexpression suppressed CUL4A expression in NSCLC cells. ***A-C,*** The overexpression of miR-363-3p decreased CUL4A mRNA and protein levels in A549 and H23 cell. ***D,*** The overexpression of miR-363-3p enhanced levels of TGFB1 and TIEG1 targets of CUL4A in A549 and H23 cells, which was ***E,*** quantitatively analyzed. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

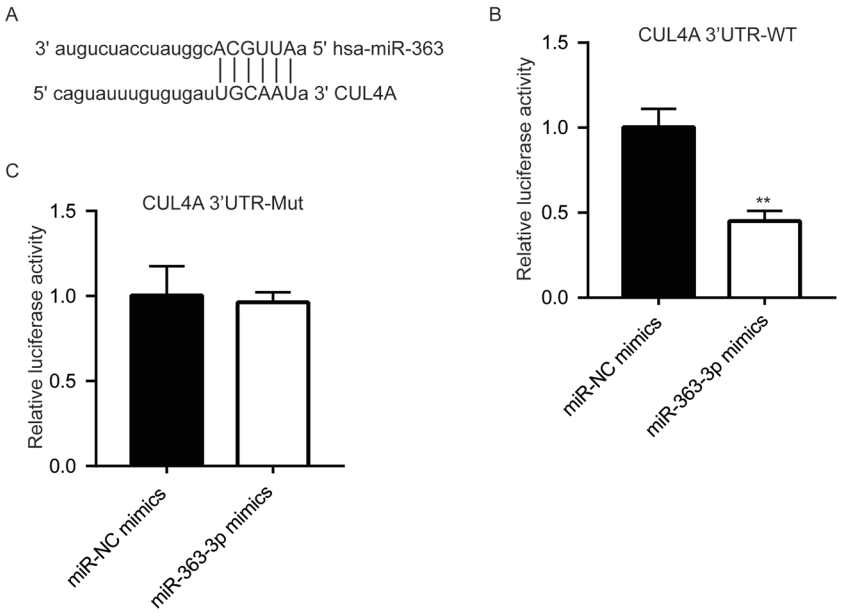

Figure 5. CUL4A was revealed to represent a target gene of miR-363-3p in NSCLC cells. ***A,*** CUL4A 3'UTR exhibited complementary sequences to miR-363-3p. ***B,*** Transfection with miR-363-3p mimics decreased the relative Luciferase activity of A549 cells transfected with CUL4A 3'UTR-WT. ***C,*** Transfection with miR-363-3p mimics did not affect the relative Luciferase activity of A549 cells transfected with CUL4A 3'UTR-Mut. $^{**}p<0.01$.

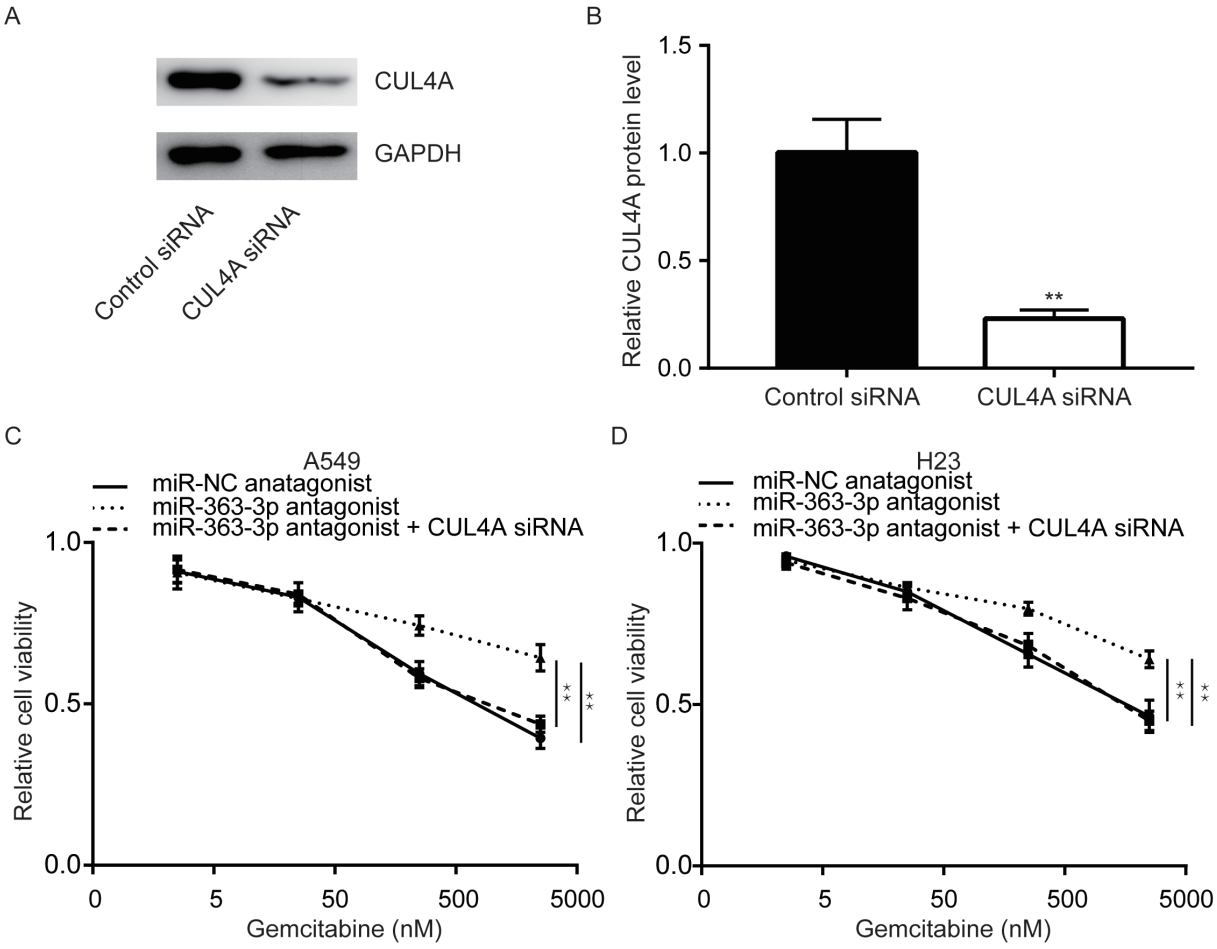

Figure 6. miR-363-3p regulated gemcitabine sensitivity of NSCLC cells via repression of CUL4A. ***A,*** The transfection of CUL4A siRNA decreased CUL4A protein expression in A549 cells, which was ***B,*** quantitatively analyzed. ***C-D,*** The inhibition of miR-363-3p decreased the sensitivity of A549 and H23 cells to increasing concentrations of gemcitabine, which was reversed by knockdown of CUL4A. $^{**}p<0.01$.

meta-analysis[32] of human lung cancer miRNA expression profiles discovered many dysregulated miRNAs in lung cancer; further investigation showed that among these miRNAs, miR-363-3p, miR-650, miR-5100 were differentially expressed between normal tissues vs. NSCLC tumor tissues and paratumor vs. NSCLC tumor tissues. In the present work, miR-650, miR-5100 (data not shown) and miR-363-3p levels were investigated using RT-qPCR and the results demonstrated that miR-363-3p levels in NSCLC tumor tissues and NSCLC cell lines were significantly decreased compared with matched normal tissues and a normal lung epithelial cell line, respectively. A study[11] suggested that miR-363-3p inhibits cell growth, leads to cell cycle arrest in S phase and induces cell apoptosis in lung adenocarcinoma by targeting PCNA. We showed that the overexpression of miR-363-3p slightly decreased the proliferation rate of NSCLC cells. However, the overexpression of miR-363-3p greatly enhanced the sensitivity of NSCLC cells to gemcitabine treatment. Furthermore, silencing of miR-363-3p was revealed to decrease the sensitivity of NSCLC cells to gemcitabine treatment. Therefore, the results of the present work suggested that miR-363-3p may represent a novel chemotherapy sensitizer in NSCLC cells. As for the future study, we have to further evaluate the function of miR-363-3p in established gemcitabine-resistant NSCLC cells, which would provide more valuable information of the development of gemcitabine resistance in NSCLC cells.

The overexpression of CUL4A has been revealed to promote the development of lung carcinogenesis in transgenic mice[33]. Furthermore, CUL4A has been demonstrated to enhance gemcitabine resistance in lung cancer cells via direct interaction with TGFB1, which subsequently induces ubiquitin-mediated protein degradation[34]. Several miRNAs have been reported to decrease CUL4A expression, which suppresses cancer progression[35,36]. In the present study, the overexpression of miR-363-3p was revealed to decrease CUL4A mRNA and protein levels, which suggested that miR-363-3p represents a negative regulator of CUL4A in NSCLC cells. In addition, enhanced expression of miR-363-3p was demonstrated to enhance TGFB1 and TIEG1 protein levels, which are regulated by CUL4A24. Bioinformatic analyses revealed that miR-363-3p exhibits complementary sequences for binding with the CUL4A 3'UTR. Furthermore, the results of the Dual-Luciferase assays further suggested that CUL4A represents a target gene of miR-363-3p in NSCLC cells. Our results indicated that miR-363-3p regulates chemotherapy sensitivity via suppression of CUL4A expression in NSCLC.

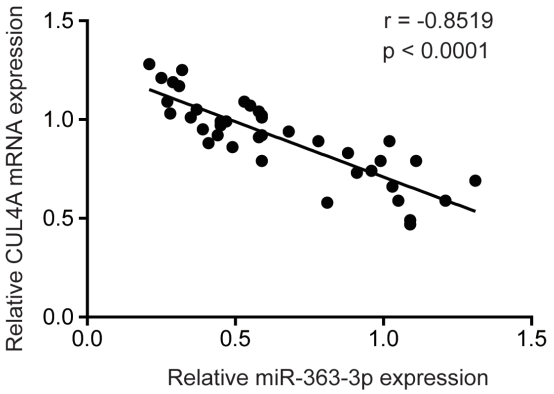

Figure 7. CUL4A mRNA expression was associated with miR-363-3p levels in NSCLC tumor tissues. Pearson's correlation analysis revealed a negative correlation between CUL4A mRNA expression and miR-363-3p levels in tumor tissues obtained from 40 NSCLC patients.

Conclusions

We revealed that miR-363-3p has an important role in the regulation of gemcitabine resistance in NSCLC. Enhanced expression of miR-363-3p was revealed to sensitize NSCLC cells to gemcitabine, which may provide novel insight for the development of future therapeutic approaches for the treatment of patients with NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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