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ORIGINAL ARTICLE

MiR-155 inhibits proliferation and invasion by directly targeting PDCD4 in non-small cell lung cancer

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Abstract

Background: MicroRNAs are often abnormally expressed in human non-small cell lung cancer (NSCLC) and are thought to play a critical role in the emergence or maintenance of NSCLC by binding to its target messenger RNA. We assessed the effects of miR-155 on cell proliferation and invasion to elucidate the role played by miR-155/*PDCD4* in NSCLC.

Methods: Quantitative reverse transcription-PCR, Western blotting, and cell counting kit-8, luciferase, and transwell invasion assays were conducted on a normal human bronchial epithelial cell line (BEAS-2B) and three NSCLC cell lines (SPC-A-1, A549, and H2170).

Results: We confirmed that miR-155 was upregulated, while *PDCD4* messenger RNA and protein levels were downregulated in NSCLC cell lines. miR-155 negatively regulated *PDCD4* at both transcriptional and post-transcriptional levels. Moreover, *PDCD4* was forecast as an assumed target of miR-155 using bioinformatic methods and we demonstrated that *PDCD4* was a direct target of miR-155 using luciferase reporter assays. Furthermore, *PDCD4* overexpression could restrain NSCLC proliferation and invasion induced by miR-155.

Conclusion: Our results collectively demonstrate that miR-155 exerts an oncogenic role in NSCLC by directly targeting *PDCD4*.

Introduction

Lung cancer is a leading cause of death globally. Non-small cell lung cancer (NSCLC) is the most frequent type, accounting for approximately 80–85%.[1,2]Squamous cell carcinoma and adenocarcinoma are the main types of NSCLC.[3]The five-year overall survival (OS) rate associated with NSCLC is a dismal 11%, in spite of enormous breakthroughs in treatment techniques.[4,5] Therefore, there is an urgent need to find novel targets important to the progression and development of NSCLC.[6]

MicroRNAs (miRNAs) are a set of non-coding RNA molecules that are small (<22 nt) yet significant in many biological processes.[7] MiRNA usually exerts its function by means of base pairing with the 3′-untranslated region (3′-UTR) of corresponding genes.[8] Accumulated evidence has shown that the alteration or dysfunction of miRNAs might play a vital role in the cell cycle, progression, apoptosis, autophagy, and migration and invasion.[9–11] Moreover, abnormal miRNA expression has been observed in the progression and development of certain types of cancer and tumors, including esophageal and lung cancers, lymphocytic leukemia, and neuroblastoma.[12–15]

The expression of the 64-kDa protein *PDCD4* is dramatically downregulated in numerous cancers, including colorectal, lung, gastric, and breast cancers, and is therefore generally recognized as a vital tumor suppressor.[16–19] To date, a set of miRNAs have been confirmed to target

PDCD4, such as miR-96 in glioma cancer, miR-4262 in hepatocellular carcinoma, and miR-499 in oropharyngeal cancer.[20–22] Although miR-155 and *PDCD4* have been shown to play distinct roles in NSCLC, the exact mechanism of miR-155/*PDCD4* is not yet clear.[16,23] Therefore, we assessed the effects of miR-155and *PDCD4* on cell proliferation and invasion in NSCLC.

Methods

Cell culture

We cultured a normal human bronchial epithelial cell line (BEAS-2B), as well as three NSCLC cell lines (SPC-A-1, A549, and H2170; Invitrogen, Carlsbad, CA, USA) in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37°C with 5% CO_2.

Tissue samples

Between 2015 and 2016, we collected a total of 26 primary NSCLC and adjacent non-cancerous tissues from the Department of Cardiothoracic Surgery, the First Affiliated Hospital of Nanjing Medical University. All patients provided informed consent before surgery. All tissue specimens were snap frozen immediately in liquid nitrogen. The patients did not undergo chemotherapy or radiotherapy before surgery. Experienced pathologists histologically determined both cancerous and adjacent non-tumor tissues. This project passed Nanjing Medical University ethical censorship.

Extraction of RNA and quantitative reverse transcription-PCR

We isolated total RNA from human tissues and cell lines using Trizol (Invitrogen). We performed quantitative real-time PCR (qRT-PCR) experiments on an ABI 7500 version of a fast real-time PCR system (Stratagene, La Jolla, CA, USA) with a high-specificity miR-155 qRT-PCR detection kit (Stratagene), in accordance with the manufacturer's protocol. U6 small nuclear RNA was chosen as a built-in control. A $2\bigtriangledown$CT method was applied to calculate the relative gene expression level.

Plasmid construction

We obtained miR-155 mimics, anti-miR-155 mimics, and their negative control oligonucleotides (miR-155 NC and anti-miR-155 NC) from RiboBio (Guangzhou, China). We then amplified 3′-UTR of PDCD4 messenger RNA utilizing the follow primers: forward 5′-GAATCTAGAATATAAGAACTCTTGCAGTC-3′ and reverse 5′-CTTCTAGAACCAGGTTCATTTTCC-3′. The PCR products obtained after amplification were implanted into the pGL3 control vector (Promega, Madison, WI, USA). A fast mutation kit (NEB, Ipswich, Canada) was then used for mutation experiments. Finally, we inserted *PDCD4* into the specific region of pcDNA 3.1and pcDNA3.1-*PDCD4* was ultimately constructed.

Cell transfection

We divided the transfected cells into seven groups in accordance with the treatment applied: (i) miR-155, (ii) miR-155 NC, (iii) anti-miR-155, (iv) anti-miR-155 NC, (v) *PDCD4* 3′-UTR-wild, (vi) *PDCD4* 3′-UTR-mut, and (vii) pcDNA3.1-*PDCD4*. We performed all transfection experiments using Lipofectamin 2000 (Invitrogen). The selection of cells transfected with miR-155 or PDCD4 was carried out using G418 and finally, we obtained the stable transfected cells.

Western blot

Total proteins were extracted from cultured cells using a RIPA Lysis Buffer (Beyotime, Shanghai, China). A dismembrator was used to homogenize 50–100 mg of tissues to extract proteins. Protein concentration was detected using a BCA Protein Assay Kit (BioRad, Hercules, CA, USA). Equal doses of protein were obtained from samples and were transfected onto polyvinylidene fluoride membrane after sodium dodecyl sulfate-polyacrylamide gel electrophoresis integration. The membranes were immersed in tris-buffered saline plus tween 20 with 5% non-fat milk for two hours. The membranes were then incubated with diluted primary antibody against *PDCD4* (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:400 overnight at 4°C and then with the secondary antibody for 1 hour at room temperature. The signals were visualized using ECL reagents (Pierce Biotechnology, Rockford, IL, USA).

Cell counting kit-8 and tumor formation test

Cell proliferation was evaluated using cell counting kit-8 assay (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan). We seeded the cells into 96-well plates at 2×10^3 cells per well. The proliferation rate was measured at zero, 24, 48, and 72 hours after cell transfection. A microplate reader (BioRad) was used to determine the absorbance at 450 nm. BALB/C nude mice aged five weeks were purchased from the Experiment Animal Center of Nanjing Medical University. The Institutional Animal Care and Treatment Committee of Nanjing Medical University approved all animal protocols. We gathered the A549 cells during the logarithmic phase. Cells were washed twice using phosphate buffered saline and resuspended at a concentration of 2×10^7 cells/mL. The female nude mice were

then subcutaneously injected with 0.1 mL of the suspended cells at either side of their flank. The size of the tumor was measured twice a week.

Luciferase test

We seeded the cells into 24-well plates with 2×10^5 cells per well. Cells were co-transfected 24 hours later with *PDCD4* 3′-UTR wild or *PDCD4* 3′-UTR mut and anti-miR-155 or anti-miR-155 NC. We harvested the cells 48 hours after transfection and treated them using a Dual-Luciferase Reporter Assay Kit (Promega) following the manufacturer's instructions.

Transwell invasion assay

Cell transfection was conducted with miR-155, miR-155 NC, and pcDNA3.1-*PDCD4*, respectively. The transfected cells (2×10^5) were placed in transwell chambers pre-coated with 20 μg Matrigel. The medium containing 10% fetal bovine serum located in the lower chamber was treated as chemoattractant. Non-invading cells above the membrane were scraped off after 24 hours of incubation. The invasive cells that were attached to the bottom were tinted with 0.05% crystal violet. We then counted the number of invaded cells under an inverted microscope at ×200 magnification. Each assay was carried out in triplicate.

Statistical analysis

All statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA). Mean values ± standard deviations were presented in all data. A student's *t*-test was applied to assess differences. A P value of < 0.05 was considered statistically significant.

Results

MiR-155 was upregulated in non-small cell lung cancer (NSCLC) cell lines, while PDCD4 messenger RNA and protein levels were downregulated

We tested messenger RNA (mRNA) and protein in *PDCD4* and miR-155 expression in four cell lines: a normal human bronchial epithelial cell line (BEAS-2B) and three NSCLC cell lines (A549, H2170, and SPC-A-1). The level of miR-155 expression in normal cells was lower than in NSCLC cell lines (Fig 1a). Inversely, the *PDCD4* mRNA level was higher (Fig 1b). Western blot results demonstrated a higher level of *PDCD4* protein in normal cells than in cancer cell lines (Fig 1c,d). These results indicated notable upregulation of miR-155 expression in NSCLC cell lines and downregulation of *PDCD4* mRNA and protein levels.

Downregulation of PDCD4 and negative correlation with miR-155 expression in NSCLC tissues

To further prove that miR-155 negatively regulates *PDCD4* not only at a transcriptional level but also at a post-transcriptional level, we performed qRT-PCR and Western

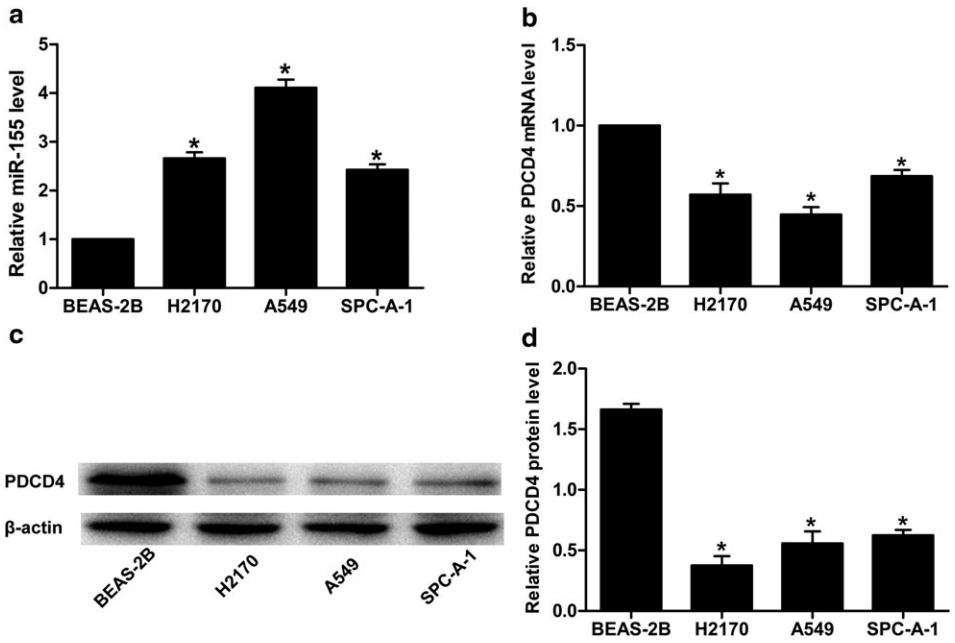

Figure 1 Relative (**a**) miR-155 expression, (**b**) *PDCD4* messenger RNA (mRNA) and (**c**) *PDCD4* protein levels in three non-small cell lung cancer cell lines (A549, H2170, and SPC-A-1) and a normal human bronchial epithelial cell line (BEAS-2B). U6 small nuclear RNA, glyceraldehyde 3-phosphate dehydrogenase, and β-actin were used as internal controls, respectively. (**d**) Relative grayscale values of Western blotting were calculated to analyze the *PDCD4* protein levels in the four cell lines (*$P < 0.05$).

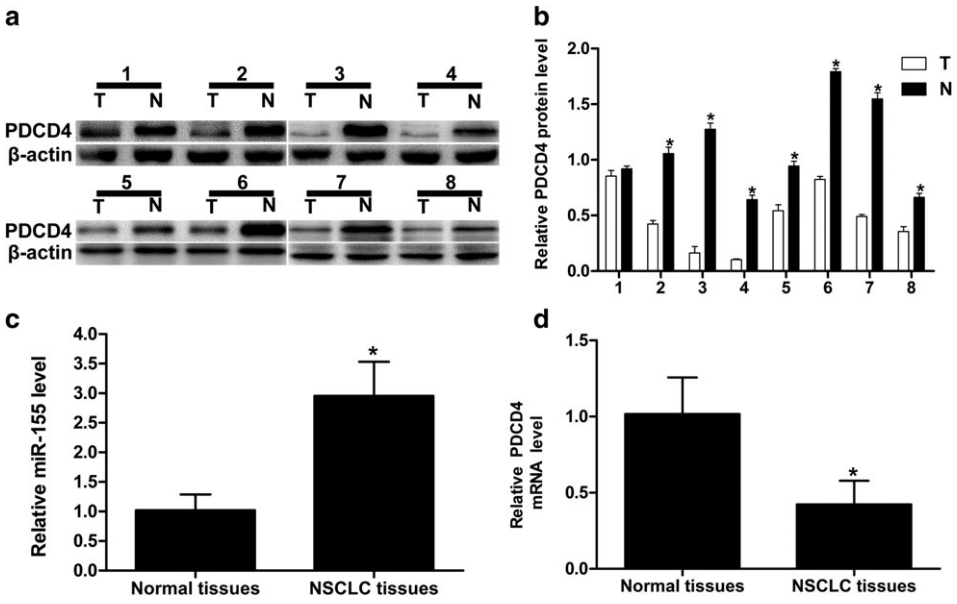

Figure 2 *PDCD4* protein levels in representative examples of eight out of 26 matched tumor tissues/normal (T/N) were analyzed via (**a**) Western blotting and (**b**) by calculating the grayscale Western blot values; β-actin was used as an internal control. (**c**) Relative miR-155 expression and (**d**) average level of *PDCD4* messenger RNA (mRNA) in non-small cell lung cancer (NSCLC) (n = 26) and adjacent non-tumor tissues (n = 26) assessed by quantitative reverse transcription (qRT)-PCR. U6 small nuclear RNA and glyceraldehyde 3-phosphate dehydrogenase were used as endogenous controls, respectively (*P < 0.05).

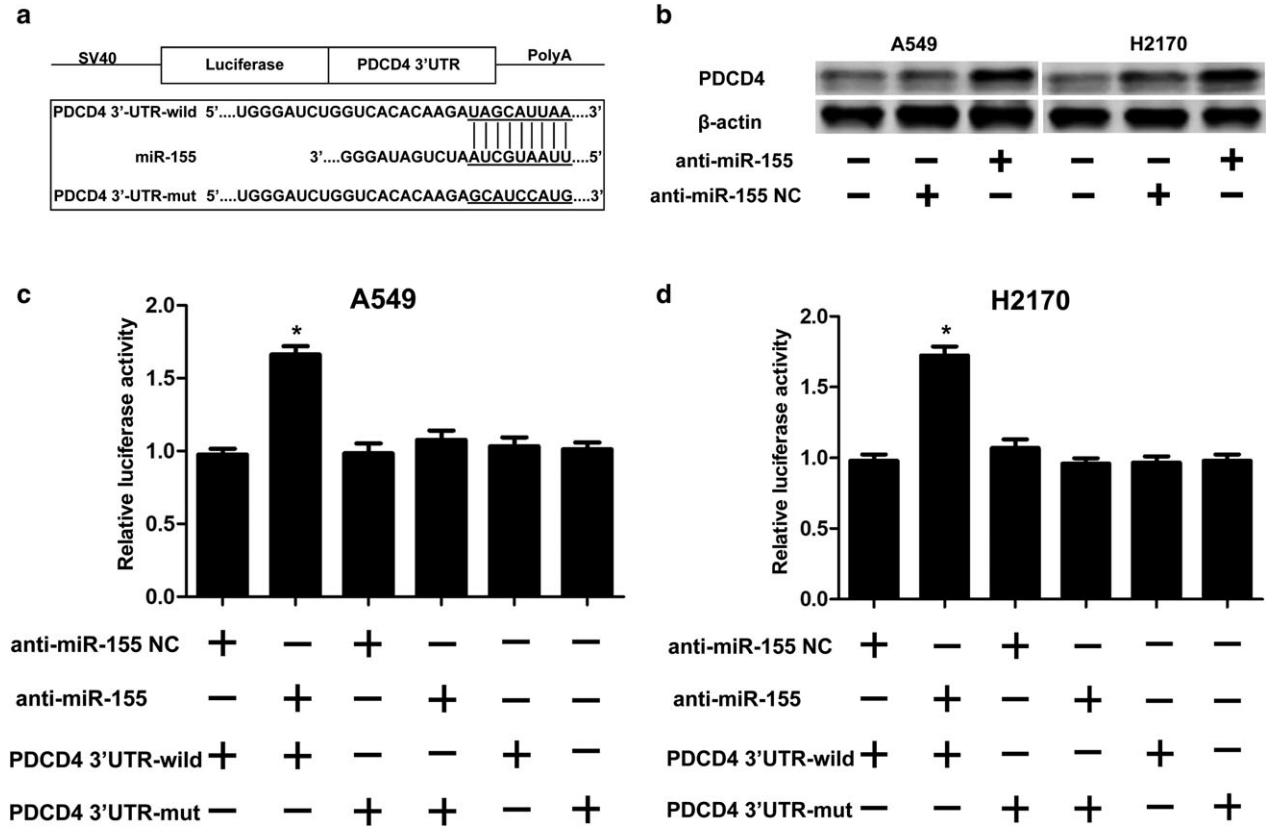

Figure 3 *PDCD4* was identified as a direct target of miR-155 in non-small cell lung cancer cell lines. (**a**) The predicted *PDCD4* 3′-untranslated region (UTR)-wild and *PDCD4* 3′-UTR-mut binding sequences in miR-155. (**b**) *PDCD4* protein levels were analyzed by Western blot. β-actin was used as an internal control. After co-transfection with miR-155 NC or miR-155 and *PDCD4* 3′-UTR-wild or *PDCD4* 3′-UTR-mut (reporter vectors), the luciferase activity in (**c**) A549 and (**d**) H2170 cells was analyzed. The measured luciferase activity was normalized to renilla luciferase activity (*P < 0.05).

blotting to analyze *PDCD4* mRNA, *PDCD4* protein, and miR-155 expression in resected tumors and adjacent normal tissues in 26 NSCLC patients. The level of *PDCD4* protein was notably lower in the tumor tissues than in the corresponding normal tissues (Fig 2a,b). Analogously, low levels of *PDCD4* mRNA were observed in the tumor tissues compared to the adjacent normal tissues (Fig 2d). In contrast, we detected that miR-155 was upregulated in NSCLC tissues compared to normal tissues (Fig 2c). This in vivo data further indicated that miR-155 negatively regulates *PDCD4* at both transcriptional and post-transcriptional levels.

PDCD4 is a direct target of miR-155

We presented the 3′-UTR of *PDCD4* and its possible binding site with miR-155 based on the bioinformatics yielded by TargetScan and miRanda (Fig 3a). We then conducted Western blotting and a luciferase test. Western blotting

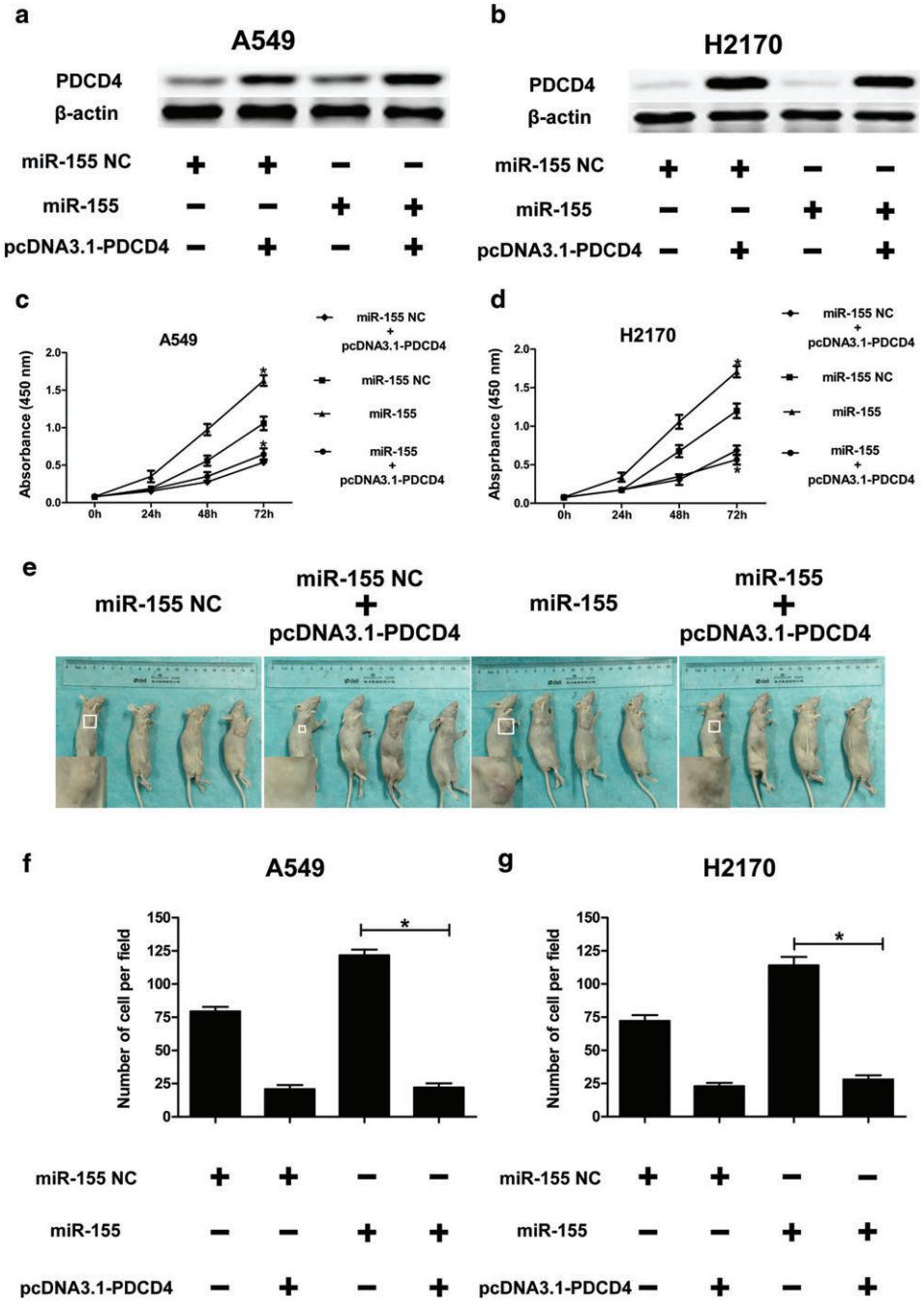

Figure 4 *PDCD4* overexpression restrains proliferation and invasion induced by miR-155. After co-transfection with miR-155 NC or miR-155 and pcDNA3.1-*PDCD4*, the *PDCD4* protein level was analyzed by Western blot in (**a**) A549 and (**b**) H2170 cells. β-actin was used as an internal control. Cell growth activity after co-transfection with miR-155 NC or miR-155 and pcDNA3.1-*PDCD4* was assessed using CCK-8 assays in (**c**) A549 and (**d**) H2170 cells (*$P < 0.05$). (**e**) Six weeks after injecting the mice with miR-155 NC, miR-155 NC + pcDNA3.1-*PDCD4*, miR-155, or miR-155 + pcDNA3.1-*PDCD4* group cells (2 × 106 A549 or H2170 cells), the nude mice were all sacrificed to compare the tumor volume in the four different groups (*$P < 0.05$). The average numbers of (**f**) A549 and (**g**) H2170 cells penetrating the transwell membrane were analyzed using transwell invasion assay (*$P < 0.05$).

demonstrated that *PDCD4* was distinctly upregulated in H2170 and A549 cells after transfection with anti-miR-155 mimic (Fig 3b). Moreover, we further conducted a luciferase reporter test to verify whether the 3′-UTR of *PDCD4* was a functional target of miR-155 in NSCLC. pGL3-*PDCD4* 3′-UTR wild and pGL3-*PDCD4* 3′-UTR mut were established (Fig 3a). When co-transfected with anti-miR-155, significantly increased luciferase activity containing *PDCD4* 3′-UTR was observed in H2170 and A549 cells (Fig 3c,d). Thus, we concluded that miR-155 combined with site of the 3′-UTR sequence of *PDCD4* regulates the *PDCD4* level and *PDCD4* serves as a direct target of miR-155.

Upregulated expression of PDCD4 could restrain the tumor promoting effect of miR-155 in NSCLC

Western blotting, CCK-8 testing, tumor formation in nude mouse models, and transwell invasion assay were performed in order to further explore the correlation between *PDCD4* and miR-155. After miR-155 NC or miR-155 were co-transfected with pcDNA3.1-*PDCD4*, the H2170 and A549 cells all displayed high *PDCD4* protein levels (Fig 4a, b). After CCK-8 testing and transfection with pcDNA3.1-PDCD4, the absorbance value in H2170 and A549 cells was reduced, but after co-transfection with miR-155 we did not observe any growth (Fig 4c,d). Furthermore, we set up four types of nude mouse models by injecting A549 cells into either side of the flank of female nude mice. Our results demonstrated that the tumor was reduced after transfection with pcDNA3.1-*PDCD4*, but co-transfection with miR-155 did not reverse this phenomenon (Fig 4e). Regarding transwell invasion assay, after transfection with miR-155, the average number of H2170 or A549 cells penetrating the transwell membrane increased. However, the average number of transwell cells after co-transfection with pcDNA3.1-*PDCD4* and miR-155 reduced, which meant that the invasion effect of miR-155 in NSCLC was abrogated (Fig 4f,g). Overall, these results imply that overexpression of *PDCD4* restrained the tumor-promoting effect of miR-155 in NSCLC.

Discussion

Accumulating evidence suggests that miRNAs function as either tumor suppressors or oncogenes by regulating various biological processes of cancer cells, such as cell proliferation, apoptosis, migration, and invasion.[24,25] Numerous studies have described the effect of miR-155 in regulating the progression of varied tumors.[26,27]There has been a trend to define miR-155 as a marker of solid and hematological malignancies for diagnosis and prognosis;[28]however, the exact role miR-155 plays in the occurrence and progression of NSCLC remains unknown. Therefore, illuminating the molecular mechanism in the occurrence and prognosis of NSCLC is of great importance.

We demonstrated that miR-155 is upregulated in NSCLC cell lines, while *PDCD4* mRNA and protein levels are downregulated. Furthermore, our results showed that *PDCD4* was downregulated in NSCLC tumor tissues and was negatively correlated with miR-155 expression. Luciferase reporter testing revealed that miR-155 exerts its tumor-promoting effect by directly targeting *PDCD4*. *PDCD4* overexpression reversed the malignant phenotypes of NSCLC, thus it may be a potential target for NSCLC treatment. In accordance with the results of previous studies, we confirmed that *PDCD4* is a tumor suppressor.[29,30] *PDCD4* suppresses cell invasion in colon cancer by inhibiting the expression of mitogen-activated protein kinase. It has also been reported that *PDCD4* could inhibit cell invasion in breast cancer by suppressing metalloproteinase 2.[31,32] These results indicate that emphasis should be placed on an exploration of the relationship between *PDCD4* and miR-155, which may cast light on the pathogenesis of NSCLC.

In conclusion, our results demonstrate the effects of miR-155 on multiplication and invasion by targeting *PDCD4* in NSCLC. miR-155 plays a crucial role in NSCLC tumorigenesis and could represent a potential NSCLC treatment strategy. miR-155 and *PDCD4* (protein and mRNA) have contrasting expression levels in NSCLC tissues and cell lines. *PDCD4* is a functional target for miR-155 and regulates proliferation or invasion by targeting *PDCD4* in NSCLC. Therefore, our data indicate that downregulating miR-155 or upregulating *PDCD4* could be potential treatments strategies for NSCLC.

Disclosure

No authors report any conflict of interest.

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