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Original Paper

MicroRNA-21 (miR-21) Post-Transcriptionally Downregulates Tumor Suppressor PDCD4 and Promotes Cell Transformation, Proliferation, and Metastasis in Renal Cell Carcinoma

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Key Words
MiR-21 • PDCD4 • Metastasis • RCC • EDU

Abstract
Objectives: MiR-21 induces neoplastic transformation, cell proliferation, and metastasis and downregulates programmed cell death4 (PDCD4) in some cancers. The aim of this study was to investigate the roles and interactions of PDCD4 and miR-21 in human renal cell carcinoma (RCC). ***Materials and Methods:*** A total of 32 paired tumor and normal tissue specimens from RCC patients as well as three renal cancer cell lines (786-O, A498, caki-1) and one normal epithelial kidney cell line (HK-2) were studied. The expression levels of PDCD4 (protein and mRNA) and miR-21 were examined by Western blot analysis and by qRT-PCR and luciferase reporter assays. Furthermore, we transfected 786-O cells with pre-miR-21 (mimics) and anti-miR-21 (inhibitor) and then again analyzed the expression of PDCD4 protein and mRNA, and determined cell proliferation and transformation capabilities by EDU and soft agar colony formation assay. ***Results:*** MiR-21 expression was significantly upregulated in RCC, metastatic RCC specimens and renal cancer cell lines (A498, 786-O, caki-1) compared to normal non-metastatic RCC specimens and HK-2 cells ($P<0.05$). In contrast, PDCD4 protein expression significantly decreased ($P<0.05$), whereas PDCD4 mRNA expression remained unaltered ($P>0.05$). Moreover, we observed a significant reduction in PDCD4 protein levels in miR-21mimic-transfected cells, but a significant increase in miR-21inhibitor-transfected cells ($P<0.05$), whereas PDCD4 mRNA was practically unaltered ($P>0.05$). Furthermore, miR-21mimic-transfected cells exhibited increased cell proliferation and transformation capacity

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according to EDU analysis and soft agar formation assay, whereas miR-21inhibitor-transfected cells exhibited the opposite phenomenon($P<0.05$). ***Conclusions:*** MiR-21 not only promoted cancer cell hyperplasia and contributed to tumor cell transformation and metastasis, but also post-transcriptionally downregulated PDCD4 protein expression. PDCD4 and miR-21 expression levels potentially play an important role in renal cell cancer.

Introduction

MicroRNAs (miRNAs) are non-protein-coding smallRNAs of approximately 19–25 nucleotides (nt) in length that are cleaved from 70 to 100 nt-long hairpin pre-miRNA precursors by the enzyme Drosha [1, 2].MiRNAs bind to complementary sequences in the 3'-untranslated regions of their target mRNAs and induce mRNA degradation or translational repression [3].Recent evidence has shown that abnormal expression levels of miRNAs are associated with a variety of human cancers, and that they play crucial roles in cell proliferation,differentiation and apoptosis [4].Volinia et al. studied the miRNA expression pattern in solid cancers (2,532 samples, 31 cancer types, 120 miRNAs including miR-21), and they found that miR-21 was highly expressed in 31 solid cancers, including stomach, prostate, head and neck, esophagus, glioblastoma, neuroblastoma, cholangiocarcinoma, breast, lung, colorectal, and pancreatic cancer [5-7].Chan et al. reported evidence indicating that increased expression of miR-21 is associated with carcinogenesis, including inhibition of apoptosis [8], promotion of cell proliferation [9] and stimulation of tumor growth [10]. Moreover, Florin et al. suggested that miR-21 is overexpressed in human cholangiocarcinoma (CCA), and reported that it was 95% sensitive and 100% specific in diagnosing human CCA [11].

PDCD4 was described by Colburn and colleagues as a new tumor suppressor gene, its overexpression being sufficient to inhibit neoplastic transformation [12, 13]. Studies in cultured ovarian cancer cells, for example, suggest that PDCD4 suppresses proliferation and cell cycle progression and induces apoptosis [14]. Furthermore, PDCD4 plays an important role in suppressing tumorigenesis by regulating several other genes involved in related processes including apoptosis, cell cycle, and cell proliferation. Acting via the MA-3 domain, PDCD4 acts as a translation inhibitor, thereby influencing protein patterns in the cells. Additionally, PDCD4 has been shown to be regulated by a diverse set of molecules, including topoisomerase-inhibitors [15], COX-2 inhibitors [16], Ak t[17, 18],and various mitogens [19]. Recently, down-regulation of PDCD4 expression has been identified in various human solid neoplasias(human colon cancer, ovarian cancer, lung cancer, primary pancreatic cancer, and glioma) [20].

Through bioinformatic analysis, it has been demonstrated that PDCD4 contains a miR-21 binding site and acts as a tumor suppressor through the regulation of various aspects associated with cancer progression, such as cell proliferation, invasion, metastasis, and neoplastic transformation [21-23]. It has been shown that knock-down of miR-21 upregulates PDCD4 expression leading to increased apoptotic cell death in glioblastomacells [8, 24] and suppresses invasion and metastasis in colorectal cancer cells [22] and esophageal squamous cell carcinoma [25]. However, relatively little is known regarding the underlying mechanisms through which miR-21 regulates PDCD4 expression in renal cancer. In this study, we aimed to: (a) determine the role of miR-21 and PDCD4 in human RCC, and (b) elucidate the interactions between PDCD4 and miR-21 in human RCC.

Materials and Methods

Ethics Statement

Tissue specimens (32 tumor and 32nontumorous tissue) from RCC patients were obtained and histologically confirmed by a pathologist at the First Affiliated Hospital of Dalian Medical University (Dalian,

China). Written informed consent was obtained from all patients and the study was approved by the Ethics Committee of Dalian Medical University on Human Research (Approvalnumber:DYH201202016). All samples were derived from patients who had not received adjuvant treatment including radiotherapy or chemotherapy prior to surgery in order to eliminate potential treatment-induced changes to gene expression profiles. After excision, tissue specimens were immediately frozen in liquid nitrogen for subsequent analysis.

Cell lines and cell culture

Human cell lines (786-O, A498, caki-1 and HK-2) were obtained from Xiehe Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China) as a commercial source. Normal renal HK-2 cells were cultured as a monolayer in Keratinocyte Serum Free Medium (K-SFM) supplemented with 0.05 mg/mL bovine pituitary extract (BPE), 5 ng/mL human recombinant epidermal growth factor (EGF) (Life Technologies/ Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 50 mg/mL penicillin and 50 mg/mL streptomycin (Invitrogen). All cell lines were maintained in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Subconfluent A-498cells (60–70% confluent) were treated with genistein (25 mM; Sigma Aldrich Corp., St Louis, MO) dissolved in DMSO; and vehicle-treated cells served as controls. Fresh genistein was administered everyday along with a change of medium, and the cells were incubated for 4 days.

Western blot

Total proteins were extracted from tissues usinga total protein extraction kit (Keygen, Nanjing, China) according to the manufacturer's recommendations. The concentrations of total proteins were measured using a BCA Protein Assay Kit (Keygen). A total of 80μg protein was separated using SDS-PAGE and transferred onto PVDF membranes; the membranes were then blocked in 5% fat-free milk at room temperature for 2h. After incubation withrabbit or goat primary antibodies against PDCD4 (Abcamab80590, Cambridge, UK) at a dilution of 1:10,000 or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200 at 4°C overnight, the membranes were probed with goat anti-rabbit or mouse anti-goat secondary antibodies at a dilution of 1:5,000at room temperature for 2h. The signals were detected using a Super ECL plus Kit (Keygen) determined by quantitative analysis using UVP software.The ratio of IOD_{PDCD4}/IOD_{GAPDH} indicated the relative expression of PDCD4 protein.

Total RNA isolation and cDNA synthesis

TRIzol reagent (CWbio. Co. Ltd., Beijing, China) was used to isolate total RNA from the snap frozen tissues. The isolated RNA was treated with DNase I (Invitrogen). The RNA concentration and purity were determined using a NanoDrop® ND-1000 (Nanodrop Products, Wilmington, DE). The ratio of 28S/18S was analyzed by GlykoBandscan 5.0. RNA quality and quantity were determined spectrophotometrically at 260 and 280 nm, respectively. Reverse transcription of RNA was performed using the NCodemiRNA First-Strand cDNA Synthesis Kit (Invitrogen, Cat#: MIRC-50).

Primer design

For miRNA-21 the primer sequence was 5-TAGCTTATCAGACTGATGTTGA-3. For U6, the sense primer sequence was 5-CTCGCTTCGGCAGCACA-3 and the antisense primer sequence was 5-AACGCTTCACGAATTTGCGT-3. For PDCD4, the sense primer sequence was 5-AGGCCGAGGTGGGCGGATCACTTGA-3andtheantisenseprimersequencewas5-GCCACCATGCCTGGCTACT-3. For GAPDH, the sense primer sequence was 5-CCTCTGACTTCAACAGCGACAC-3and the antisense primer sequence was 5-TGGTCCAGGGGTCTTACTCC-3.

Quantitative real time PCR (qRT-PCR)

qRT-PCR was performed usingthe Light Cycler 2.0 Real-Time PCR System (Roche Germany) in a total volume of 20μL in glass capillaries containing 2 μL of cDNA, 0.8 μL of each primer, and 10 μLof Light Cycler TaqMan Master Mix (Invitrogen, Cat#:MIRC-50). The PCR reaction for the miR-21 gene was initiated using a 10 min denaturation step at 95°C followed by termination with a 30s cooling step at 40°C. The cycling protocol consisted of denaturation at 95°C for 15 s and annealing at 60°C for 60 s; 40 cycles. Fluorescence detection was performed at the end of each extension step. The PCR reaction for the PDCD4 gene was initiated with a 10-min denaturation at 95°C. Amplification was carried out for 40 cycles of 15 s at 95°C and

60 s at 60°C. An additional extension step of 5 min at 72°C was added following the completion of 40 cycles. All PCR reactions were performed in duplicate. The PCR products were confirmed by melting curve analysis. We used the mathematical delta-delta method(ratio=$2^{-\Delta\Delta CT}$) developed by PE Applied Biosystems (Perkin Elmer) to compare relative expression results between treatments in qRT-PCR.

MiRNA-21 target prediction

Two independent online databases, TargetScan and miRanda, were used to predict miR-21 targets.The genes predicted by TargetScan with no fewer than two miR-21 binding sites and at least one evolutionarily conserved site, and those predicted by miRanda with scores higher than the averagescore of all miR-21 targets were selected as the miR-21 candidate targets.

Luciferase dual-reporter assays

786-O cells were seeded in a 96-well clusters at 50% to 60% confluence. After 24 hours, cells were transfected with miR-21 expression vector, anti-miR-21 vector, control vector, or negative control. Cells were co-transfected with the wild-type or mutant 3'-untranslated region(3'-UTR) of PDCD4 mRNA. Transfections were performed using Fugene(Promega, Madison, WI).Cells were collected 48 hours after transfection, and Firefly and renilla luciferase activities were measured with a Dual-LuciferaseReporter System (Promega).

RNAi assay

786-O cells were incubated in a six-well tissue culture dish without antibiotics for 24 h prior to transfection, when they had reached 60–80% confluence. Negative control (NC) siRNA, specific miR-21 inhibitor and mimic siRNA transfection reagent complexes were mixed with Lipofectamine2000 (Invitrogen) according to the manufacturer's recommendations and then added to the cells. After 6h at 37°C, the medium was changed and the cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum for various times. Silencing of miRNA21 and PDCD4 was determined by qRT-PCR and Western blot.

EDU proliferation assay

Transfected 786-O cells were plated in 24-well plates at 4×104 cells/well, allowed to adhere, washed with PBS, and incubated in serum free RPMI containing 10μmol/L 5-ethynyl-2' deoxyuridine(Guangzhou RiboBio Co., Ltd, Guangzhou, China) for 2h. The cells were then washed with PBS, fixed, and permeabilized in PBS containing 2% formaldehyde, 0.5%Triton X100, and 300 mmol/L sucrose for 15 min. After washing with PBS, cells were blocked using 10% FBS in PBS for 30 min, and incorporated EdU was detected by incubation with afluorescentazide coupling solution (Apollo; Guangzhou RiboBio Co., Ltd) for 30 min. The cells were washed three times with PBS containing 0.05% Tween20 (PBST), incubated with the DNAstaining dye Hoechst 33342 for 30 min, and washed in PBS. Images were captured using a fluorescent microscope, and the average nuclear fluorescent intensity was calculated from at least 50 non-S phase cells randomly selected in five different fields of view.

Soft agar colony formation assay

A bottomlayer (0.6% low-melt agarose) was prepared with RPMI 1640medium containing 10% fetal bovine serum, 100 U/mLpenicillin, and 100 mg/mLstreptomycin. A top layer (0.3%low-melt agarose) was prepared with the same RPMI 1640medium as described above plus 5000 of the indicated cells.Plates were incubated at 37.8°C in 5%CO_2 in a humidifiedincubator for approximately 2 weeks. The plates were then scannedand photographed, and the number of colonies was quantifiedusing Quantity one v.4.0.3 software (Bio-Rad, Hercules, CA).

Statistical analysis

SPSS 13.0 software was employed for the analysis of all data. Data were expressed as the mean±SD, and Student's paired t-test and ANOVA were used to determine the significance of the difference in multiple comparisons. An asterisk represents a P value < 0.05, which indicated a statistically significant result.

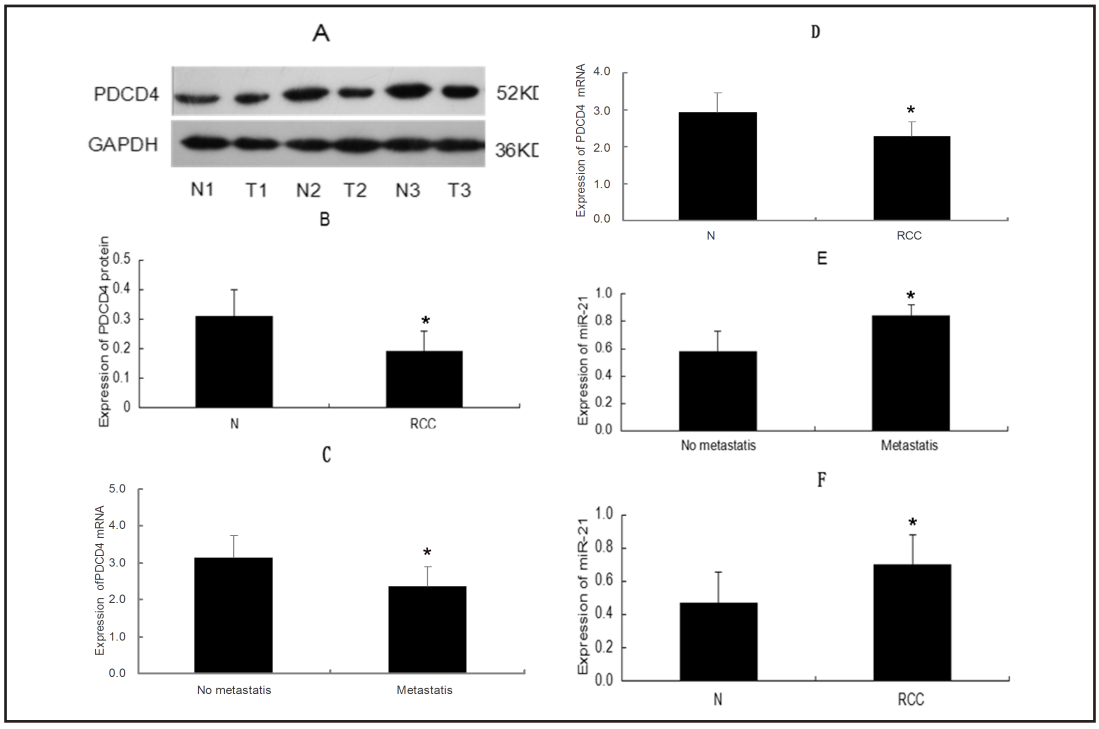

Fig. 1. Expression of PDCD4 and miR-21 in 32 RCC and non-cancerous tissue specimens.(All experiments were repeated three times). A: Representative picture of Western blot analysis of PDCD4 expression in RCC and normal tissues. B: The statistical histogram of the density of PDCD4 protein from the 32 paired tissue samples. Data are presented as ratios of PDCD4 to corresponding GAPDH. $P<0.05$ compared with that of non-cancerous tissues. *$P<0.05$C: mRNA qRT-PCR for PDCD4. X-axis:15 metastatic RCCs and 17 non-metastatic tissues. Y-axis: PDCD4 mRNA qRT-PCR value relative to specimen N1. *P*-value, Student's unpaired *t* test.$P<0.05$. D: mRNA qRT-PCR for PDCD4. X-axis: normal and cancerous primary specimens. Y-axis: PDCD4 mRNA qRT-PCR value relative to specimen N1. *P*-value, Student's unpaired *t* test.$P<0.05$. E: qRT-PCR data for miR-21 in 15 metastatic RCCs and 17 non-metastatic tissues. Y-axis, miR-21 qRT-PCR values as ratios to the N1 normal specimen; *P*-value obtained by Student's unpaired *t* test $P<0.05$. *$P<0.05$. F: qRT-PCR data for miR-21 in 32 primary RCCs and 32 normal tissues. Y-axis, miR-21 qRT-PCR values as ratios to the N1 normal specimen; *P*-value obtained by Student's unpaired *t* test $P<0.05$. *$P<0.05$.

Results

Expression of miR-21 and PDCD4 in tissue specimensand relationship to tumor metastasis

Our results demonstrated that the expression of PDCD4 was significantly reduced in RCC tissues compared with non-cancerous tissues (Fig. 1 A and B). We investigated miR-21 expression in 32 paired tissue specimens, including 17 cases of non-metastatic RCC and 15 cases of metastatic RCC. Normal tissues and 17 non-metastatic RCC tissues displayed uniformly lower expression of miR-21 than the RCC tissues or the 15 metastatic RCC tissues (Fig. 1 E and F). Similarly, there was a difference in the level of PDCD4 mRNA between the RCC tissues and the metastatic RCC cases compared to the normal renal tissues and non-metastatic RCC tissues(Fig. 1C and D).

Expression of MiR-21 and PDCD4 in renal cancer cell lines

We next determined the expression levels of miR-21 and PDCD4 (mRNA and protein) in three renal cancer cell lines and one normal renal cell line (Fig. 2). In renal cancer cell lines with high endogenous miR-21 according to qRT–PCR analysis(Fig. 2C), a low level of PDCD4 protein was observed at 52kDa(Fig. 2A and B) by westernblot analysis, whereas the normal

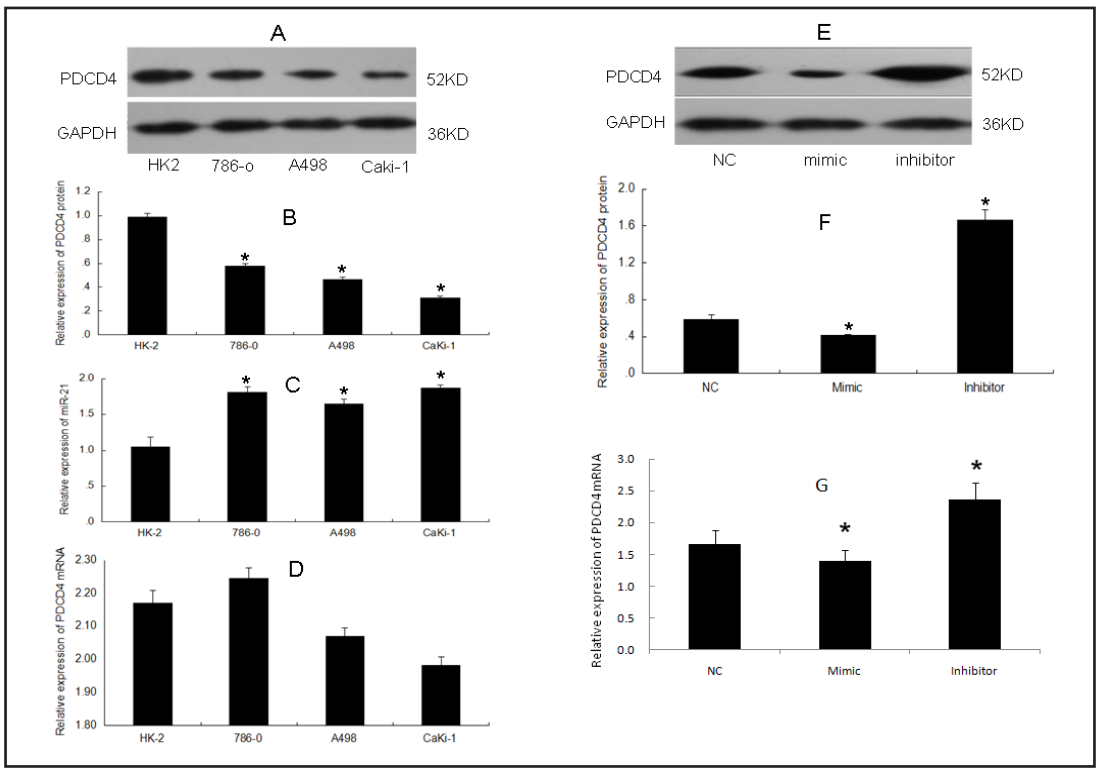

Fig. 2. Expression of MiR-21 and PDCD4 in renal cancer cell lines. (All experimentswere repeated three times). MiR-21 regulates PDCD4 expression at the post-transcriptional level. 786-O cells were transfected with 40nmol miR-21mimic, 100 nmol miR-21inhibitor and 100nmol negative control siRNA(NC). After 36h, total RNA and protein was isolated. A: Expression of PDCD4 protein in the normal renal cell line (HK-2) and in renal cancer cell lines (786-O, A498, CaKi-1), detected by westernblot. B:Relative densitometry analysis in the normal renal cell line (HK-2) and in renal cancer cell lines (786-O, A498, CaKi-1). Data are presented as ratios of PDCD4 to corresponding GAPDH. $P<0.05$ compared with that of HK-2. *$P<0.05$. C: qRT-PCR data for miR-21 in the normal renal cell line (HK-2) and in renal cancer cell lines (786-O, A498, CaKi-1). Y-axis, miR-21 qRT-PCR values as ratios to the HK-2 specimen. *$P<0.05$. D: mRNA qRT-PCR for PDCD4. X-axis: the normal renal cell line (HK-2) and renal cancer cell lines(786-O,A498,CaKi-1). Y-axis: PDCD4 mRNA qRT-PCR value relative to HK-2 specimen. $P>0.05$ E: Proteins from 786-O cells transfected with negative control siRNA(NC), miR-21mimic and miR-21inhibitor were used to detect PDCD4 by Western blott. F: Analysis of the relative density of 786-O cells transfected with negative control siRNA(NC), miR-21mimic and miR-21inhibitor. Data are presented as ratios of PDCD4 to corresponding GAPDH.$P<0.05$ compared with that of NC. *$P<0.05$. G: mRNA qRT-PCR for PDCD4. X-axis: 786-O cells were transfected with negative control siRNA, miR-21mimic and miR-21inhibitor. Y-axis: PDCD4 mRNA qRT-PCR value relative to NC specimen.$P<0.05$

renal cell line (HK-2) with low levels of miR-21 demonstrated high levels of PDCD4 protein. For PDCD4 mRNA, however, there was no significant difference (Fig. 2C).

PDCD4 is a target of miR-21

Next we aimed to find the target genes of miR-21 to explain its function. We first performed a bioinformatic screen to identify target gene candidates. We analyzed the 3′UTR binding site prediction by two independent online databases, TargetScan and miRanda. PDCD4 was selected for further experimental validation, because the complementary sequence of miR-21 was identified in the 3′-UTR of PDCD4 mRNA by miRanda analysis (Fig. 3A). To assess whether miR-21 could directly alter the expression of PDCD4, a fragment of the 3′UTR of PDCD4 mRNA (wt 3′UTR) containing the putative miR-21 binding sequence (or the mutant

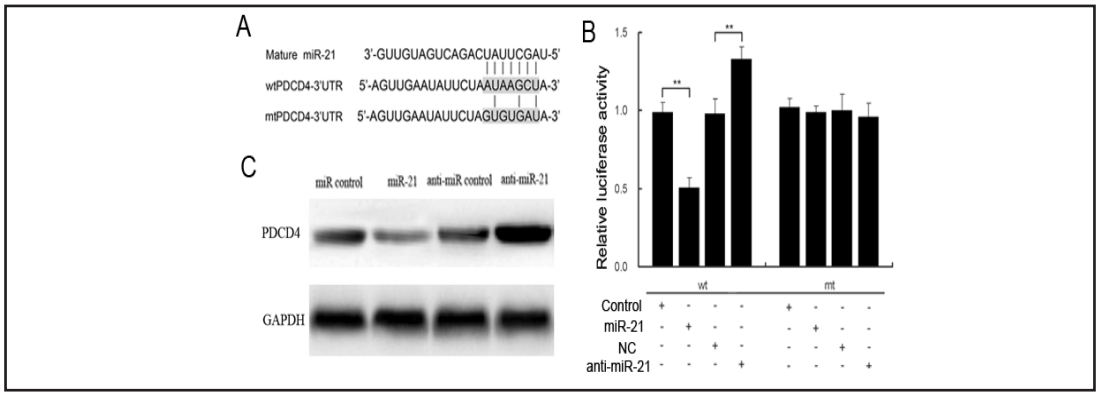

Fig. 3. PDCD4 is a target of miR-21.(All experiments were repeated three times). A. Putative binding site of miR-21 on the CKS2 3′UTR along with the mutation in the predicted seed region. B. Reporter assays on 786-O cells transfected with the reporter vectors containing either the wild type or mutated PDCD4 3′UTR. C. Protein levels of CKS2 in 786-O cells transfected with miR-21 mimic, anti-miR-21 or their nonspecific controls.

sequence, mut 3′UTR), was cloned into a luciferase reporter vector (Fig.3A). 786-O cells were then transfected with the wild type or mut 3′UTR of PDCD4 and miR-21. As shown in Fig. 3B, luciferase expression decreased when the wt 3′UTR and miR-21 were cotransfected, while the mut 3′UTR had no effect on luciferase activity. Moreover cotransfection of 786-O cells with the wt 3′UTR and anti-miR-21 reversed the decrease caused by miR-21 (Fig. 3B). Collectively, these results indicate that PDCD4 is a direct target of miR-21.

We next elucidated whether the growth-suppressing effect of miR-21 was mediated by repression of PDCD4 in 786-O cells. We first verified whether expression of PDCD4 changed in response to transfection with miR-21 or anti-miR-21. Compared to controls, expression of PDCD4 was significantly reduced by miR-21 transfection and increased by anti-miR-21 transfection (Fig.3C), indicating that miR-21 causes a reduction in PDCD4 expression in 786-O cells.

miR-21 post-transcriptionally downregulates PDCD4 protein in cultured 786-O cells

We determined whether transfection of 786-O cells with miR-21mimic or miR-21inhibitor affects PDCD4expression. In 786-O cells characterized by high miR-21 expression, down-regulation of endogenous miR-21 with miR-21inhibitor (Fig. 3) led to a significant increase in PDCD4 protein levels compared to the control according to westernblot results (Fig. 2 E, F). In contrast, there was a significant reduction in PDCD4 protein levels in miR-21mimic-transfected cells (Fig. 2 E, F). PDCD4mRNAshowed the same trend in miR-21-transfected cells (Fig. 2G).

Knockdown of miR-21 gene inhibited cell proliferation and clonogenic survival

We determined the effect of knockdown of miR-21 genes on cell viabilityand proliferative ability using an EDU assay (Fig. 4). To confirm the increased number of 786-O cells following treatment with miR-21mimic and miR-21inhibitor, cells were labeled with EdU to measure active DNA synthesis (red) and Hoechst 33342 to show all cell nuclei (blue) (Fig. 3A). According to the results of fluorescent microscopicanalysis, the mean percentage of new cells that incorporated EdU was 21.3% in the negative control siRNA group, 40.7% in miR-21mimic transfected cells, and 10.9% in the miR-21inhibitor transfected cells (Fig. 4A and B). Furthermore, we observed that the proliferative ability of 786-O cellstransfected with miR-21inhibitordecreased with increasingconcentrations of miR-21inhibitor over the range from 80 nmol/μLto 120 nmol/μLwith atime of transfection from 24h to 48h (Fig. 4C).

A colony formation assay was performed to further evaluate whether miR-21 knockdown synergistically inhibited 786-O cell transformation ability. Similarly, the colony formation

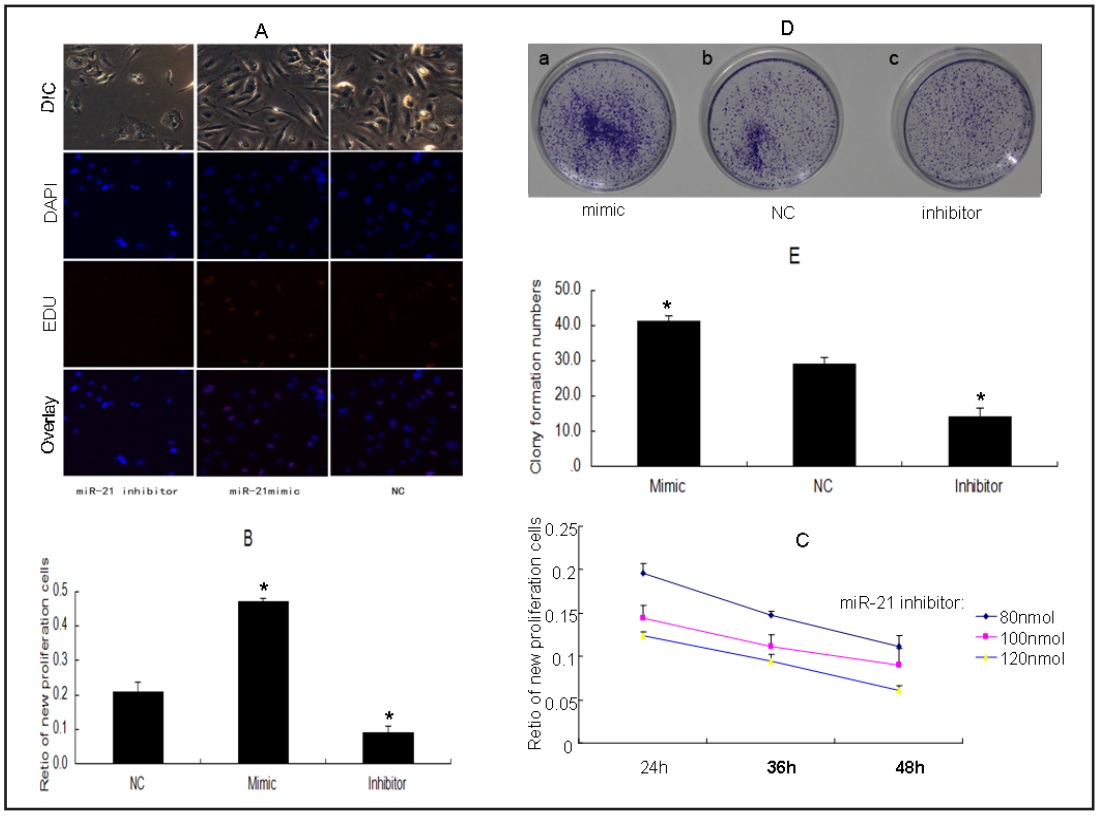

Fig. 4. MiR-21 gene knockdown inhibits cell proliferation.(All experiments were repeated three times). A: The new generation cells were detected by EDU (red). Hoechst 33342 was used for nuclear staining of all cells (blue). Overlay showsmerging of the EDU and DAPI. B: Proliferative ability data for 786-O cells transfected with negative control siRNA(NC), miR-21mimic and miR-21inhibitor. Y-axis,the cell number of EDU staining as ratios to the cell number of nuclei staining. *P*-values were obtained by Student's unpaired *t* test $P<0.05$. *$P<0.05$. C: The proliferative ability of 786-O cells transfected with miR-21inhibitor changed when the dosage of miR-21inhibitor was increased from 80 nmol to 120 nmol and the time of transfection from 24 to 48h. D: The result of colony formation assay for the 786-O cells transfected with negative control siRNA(NC), miR-21mimic and miR-21inhibitor was obtained on the 14th day. E: The colony-forming ability of 786-Ocells was significantly decreased in the miR-21 inhibitor-transfected group at 2 weeks compared with the negative control group($P<0.05$).The miR-21mimic-transfected group showed the opposite effect ($P<0.05$). *$P<0.05$.

assay demonstrated that the total number of colonies generated by miR-21inhibitor-treated cells was less compared to negative control siRNA-treated cells (NC), whereas the number of miR-21mimic-treated cell colonies was further increased compared to NC (Fig.4 D, E).

Discussion

This study is the first to indicate that the PDCD4 protein is negatively regulated by miR-21 in RCC. miRNAs are non-coding RNAs, approximately 22 nucleotides in length, which function as post-transcriptional regulators [26].MiRNAs bind to complementary sequences in the 3′-untranslated regions of their target mRNAs and regulate protein coding gene expression by inducing mRNA degradation, repressing translation or cleaving RNA transcripts in a sequence-specific manne r[2].Karginov et al. devised a direct biochemical method for miRNA target discovery that combined RNA-induced silencing complex (RISC) purification with microarray analysis of bound mRNAs. They found that examining the complete spectrum of

miR-124 targets in 293 cells yielded both a set that were down-regulated at the mRNA level, as previously observed, and a set whose mRNA levels were unaffected by miR-124a [27]. Lewis et al.indentified the targets of vertebrate miRNAsusing an algorithm called TargetScan. They found that the conserved 5′ region of mammalian microRNAs is most important for target identification [28]. A bioinformatic search revealed a conserved target site for miR-21 within the PDCD4 3'-UTR at nucleotides 228–249 [29]. Resected normal/tumor tissues of 22 colorectal cancer patients also demonstrated an inverse correlation between miR-21 and PDCD4 protein [29]. Furthermore, the results indicated that miR-21 induced invasion/metastasis in colorectal cancer cells, and PDCD4 was shown to inhibit invasion and intravasation [23, 29]. In our study, we demonstrated that the inverse correlation between miR-21 and PDCD4 protein was also observed in RCC. MiR-21expression was significantly upregulated in RCC tissues and renal cancer cell lines. In contrast, PDCD4 protein expression was significantly decreased, whereas PDCD4 mRNA expression Showing results consistent. Furthermore, we observed a significant reduction in PDCD4 protein levels and mRNA in miR-21mimic-transfected cells, but a significant increase in miR-21inhibitor-transfected cells. The results suggest that miR-21 may post-transcriptionally and negatively regulate PDCD4 by repressing translation in RCC. Furthermore, our results confirm an inverse relationship between miR-21 and PDCD4 in RCC. However, miR-21 may also directly regulate PDCD4 in RCC, since other studies in different cell types have shown that PDCD4 is a target of miR-21, and miR-21 also may indirectly regulate PDCD4 through other pathways. We confirmed that PDCD4 is the target gene of miR-21,by dual luciferase gene reporter system analysis and Western blot, in RCC cell line 786-O.

PDCD4 was initially identified as an up-regulated apoptosis-related protein, suggesting it played a role as a tumor suppressor gene [30]. Reduced PDCD4 expression has been reported in at least five human tumors (lung, brain, breast, colon, and pancreas) [21]. In our study, PDCD4 expression was significantly decreased in RCC tissues and renal cancer cell lines compared with normal renal tissues anda normal renal cell line(HK-2). Moreover, Li et al. observed that PDCD4 expression was found to be significantly associated with RCC metastasis, tumor, T-stage and tumor grade. Furthermore, decreased PDCD4 expression proved to be an independent prognostic marker for overall survival in addition to the presence of tumor metastasis and high tumor grade, and mean overall survival was significantly decreased in the low PDCD4 group compared to the high PDCD4 group [31]. All these findings demonstrate that PDCD4 is potentially not only a tumor suppressor gene, but also an invasion inhibitor in RCC. This is the first study to demonstrate the expression pattern and independent prognostic value of PDCD4 in RCC to our knowledge.

MiR-21 is one of the most prominent miRNAs and is implicated in the genesis and progression of human cancer, having been shown to be over-expressed in 31 solid tumors [5-7]. Chan et al. found that increased expression of miR-21 is associated with carcinogenesis, including inhibition of apoptosis [8], promotion of cell proliferation[9] and stimulation of tumor growth [10].Previous studies using microarrays suggested that p21 expression positively correlated with the suppression of genes that are important for cell cycle progression [32]. Zaman etal.found that cDKN1A (p21) was up-regulated after inhibition of miR-21 by cell cycle PCR array experiments,which showed an inhibitory effect on cell proliferation [33]. p21 responds to a variety of stimuliandpromotes growth-inhibitory activities depending on its ability to inhibit the activity of cyclin-dependent kinase, CDK2. Numerous studies have reported that the p21 gene has additional roles in cell cycle regulation which are independent of CDK2. These include its association with the transcription factor E2F1 and the suppression of its transcriptional activity [34]. Furthermore, p21also suppresses activity of the transcription factors STAT3 [35] and MYC [36]. In addition, p21 also promotes cell cycle inhibition through mediation of p53-dependent repression of genes such as CDC25C, CDC2, CCNB1 and BIRC5, also known as surviving [37-39]. In our present study,the results suggest that miR-21 may negatively regulate tumor suppressor PDCD4 directly or indirectly at the post-transcriptional level in RCC. Lankat-Buttgereit et al. observed that PDCD4 could regulate multiple proteins, which are involved in tumor progression, cell cycle, cell invasion,

and metastasis,in cells at both the transcriptional and translational level [40]. PDCD4 can reduce the expression of CA II at the translational level to inhibit tumor growth by decreasing bicarbonate flux of tumor cells [41]. However, PDCD4 targets several proteins not only at the translational level, but also at the transcriptional level. PDCD4 regulates uPAR expression, which mediates degradation of extracellular matrix components at the translational level, to promote tumor cell invasion and metastasis [23]. In addition, reduced levels of PDCD4 can increase the expression of E-cadherin resulting in the promotion of cell metastasis. Moreover, PDCD4 can influence cell cycle progression by regulation of CDKs, resulting in reduced cell proliferation [42]. In our study, normal renal tissues and the cell line (HK-2) displayed uniformly low expression of miR-21; in contrast, RCC tissues and cell lines (786-O, A498, and CaKi-1) displayed high expression levels. High miR-21 was associated with the presence of RCC metastasis, supporting our observation that miR-21 induced invasion-related processes. Furthermore, miR-21mimic-transfected cells exhibited increased cell proliferation and transformation capacity according to EDU analysis and soft agar formation assay, whereas miR-21inhibitor-transfected cells exhibited the opposite phenomenon. Therefore, we could assume that downregulation of PDCD4 by miR-21 may result in proliferation or metastasis in RCC. Taken together,our biological evidence suggests that targeting miR-21 may provide a good strategy to block tumor proliferation and metastasis. A correlation was found between the level of miR-21 expression and RCC metastasis and we observed that expression of PDCD4 is related to a clinical outcome such as recurrence, progression or survival [32]. From the clinical viewpoint, high expression of miR-21 and low expression of PDCD4 might be considered to be a risk factor for tumor progression, and thereby a strict systemic therapeutic strategy after surgery, such as immunotherapy, angiogenesis inhibitor drugs, chemotherapy and radiotherapy with a regular investigation might improve prognosis.

In conclusion, our present study suggests that the tumor suppressor PDCD4 is negatively regulated at the post-transcriptional level by miR-21 andthat miR-21 induces proliferation and invasion/metastasis in RCC. Taken together, the results suggest that, as an oncogenic miRNA, miR-21 plays a role not only in tumor growth, but also in tumor metastasis. This, together with our correlative results in previous clinical studies on miR-21 and PDCD4, suggests that inhibitory strategies against miR-21, or strategies interfering with the miR-21/PDCD4 interaction, or rescue of PDCD4 expression, will provide a strong rationale for therapeutic applications in renal cancer in the future.

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Disclosure Statement

The authors declare that no conflicts of interest exist.

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