miR-21 promotes migration and invasion by the miR-21-PDCD4-AP-1 feedback loop in human hepatocellular carcinoma

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Abstract. Distant migration and invasion is the main contributor to the death of cancer patients and miRNAs have been implicated in these processes. In the present study, we identified the role of microRNA-21 (miR-21) in hepatocellular carcinoma (HCC) migration and invasion and determined its underlying regulatory mechanism. miR-21 was significantly upregulated in HCC tissues and cell lines, compared with adjacent non-tumor tissues and normal hepatic cells. miR-21 upregulation was associated with the capacity of tumor migration and invasion in HCC. The expression level of miR-21 was inversely correlated with the protein expression level of a previously identified target gene, programmed cell death 4 (PDCD4). In HepG2 cells, inhibition of miR-21 expression repressed cell migration and invasion by upregulating both mRNA and protein levels of PDCD4 and downregulating key downstream signaling pathway molecules, including phospho-c-Jun, matrix metalloproteinases (MMP)-2 and MMP-9. Moreover, activation protein 1 (AP-1) could directly activate miR-21 transcription. Taken together, these results provide evidence that miR-21 promotes migration and invasion in HCC through the miR-21-PDCD4-AP-1 feedback loop, suggesting that targeting the miR-21-PDCD4-AP-1 loop may represent a promising strategy in the management of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors diagnosed in Asian countries, and is the second leading cause of cancer mortality in China. Surgical resection remains the main radical treatment of choice for HCC. HCC has poor prognosis and the 5-year survival rate after operation has been reported to range from only 36-50% (1). Like many other cancers, distant metastasis is the main contributor to poor prognosis. Migration and invasion of HCC is a multi-phase developmental process that involves changes in multi-gene signatures. Therefore, early prevention of HCC invasion and metastasis has become the primary focus of many research studies. Some molecules have been found to play an important role in HCC invasion and metastasis, such as p53 mutation (2), MDM-2 (3), telomerase (4), β-catenin (5) and VEGF (6). These molecules represent promising therapeutic targets for preventing HCC metastasis; however, their specificity and sensitivity need to be further studied.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that are 18-25 nucleotides in length and negatively regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of specific target messenger mRNAs (mRNAs), which in turn causes mRNA degradation or translational repression (7). Aberrant miRNA expression has been linked to human diseases, including cancer. As an oncogene, microRNA-21 (miR-21) was found to be overexpressed in a variety of malignancies, including cholangiocarcinoma (8), breast cancer (9), lung cancer (10), prostatic carcinoma (11) and colon carcinoma (12). miR-21 has also been implicated in multiple malignancy-related processes, including cell proliferation, apoptosis, invasion and metastasis, by downregulating the expression of specific target genes, such as phosphatase and tensin homologue (PTEN), tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4) and B-cell lymphoma 2 (Bcl-2) (13-16). Although the miR-21-PDCD4 axis has been addressed in other cancer types (19,24), the involvement of miR-21 in the migration/invasion of HCC and its underlying mechanism is not yet known.

As a previously identified target gene of miR-21, PDCD4 harbors a highly conserved binding site for miR-21 in its 3'-UTR by using bioinformatics tools. PDCD4 is a tumor suppressor that plays an important role in regulating apoptosis, invasion and metastasis (17,18). In pancreatic ductal adenocarcinoma, inhibition of miR-21 reduces proliferation and increases cell death by increasing PDCD4 (19). We hypothesized that miR-21 promoted migration and invasion of HCC by a mechanism involving PDCD4. This is the first report to demonstrate that miR-21 indirectly activates activation protein 1 (AP-1)-dependent transcription by repressing PDCD4, and stimulates

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migration and invasion in HCC. Meanwhile miR-21 transcription is directly triggered by AP-1. Together, these data suggest that the miR-21-PDCD4-AP-1 feedback loop sustains the biological effects of miR-21 in HCCs.

Materials and methods

Patients and tissue samples. Paired HCC tumor tissues and adjacent non-tumor liver tissues were collected from 16 HCC patients who underwent hepatectomy between October 2010 and March 2011 at Xiangya Hospital (Changsha, China), after obtaining informed consent and verification by an experienced pathologist. Tissue samples were frozen in liquid nitrogen immediately after resection and stored at -80°C until use. No patient in the current study received chemotherapy or radiation therapy before the surgery. The pathological differentiation was defined according to the Edmondson system. The clinical stage was defined according to the tumor-node-metastasis (TNM) staging system (6th UICC) (20). Among the patients, eight had primary HCC lesions accompanied by intrahepatic metastases, vascular invasion, or capsular invasion, and eight had no metastasis/invasion found at surgery. This study was approved by the Human Research Ethics Committee of Xiangya Hospital. Clinicopathologic characteristics of these patients, including gender, age, differentiation, metastasis/invasion, TNM staging, hepatitis-B surface antigen (HBsAg), cirrhosis, and α-fetoprotein (AFP), were collected and listed in Table I.

Cell culture and transfection. Human normal liver cells L02 and human hepatocellular carcinoma cell lines HepG2, MHCC97H, Bel7402 and Huh7 were obtained from the Cell Center at the Xiangya School of Medicine. All cell lines were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin in humidified incubator at 37°C with 5% CO_2.

The miR-21 inhibitor and PDCD4 siRNA were purchased from GenePharma (Shanghai, China). HepG2 cells were plated in 6-well plates at 50% confluence the day before transfection. The next day, 50 nM of siRNA or miR-21 inhibitor was transfected into HepG2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total-RNAs or proteins were prepared at 48 or 72 h post-transfection and used for quantitative reverse transcription (qRT)-PCR or western blot analysis, respectively. For the PDCD4 siRNA and miR-21 inhibitor combination experiments, HepG2 cells were first transfected with miR-21 inhibitor (50 nM) or inhibitor negative control (NC) (50 nM). At 48 h later, these cells were co-transfected with PDCD4 siRNA (50 nM) and miR-21 inhibitor (50 nM) or control oligos for another 72 h. Cells were used for transwell assays or qRT-PCR/western blot analyses.

qRT-PCR analysis of miR-21 and PDCD4, matrix metalloproteinases (MMP)-2, and MMP-9 mRNA expression. Total-RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Expression of miR-21 was determined by the NCode EXPRESS SYBR-GreenER miRNA qRT-PCR kit (Invitrogen), and normalized to U6 snRNA using the $2^{-\Delta\Delta Ct}$ method (21). PDCD4, MMP-2 and MMP-9 mRNA

Table I. Clinicopathologic characteristics of the HCC patients.

Characteristics	
Age, years (mean ± SD)	49.9±10.5
Gender (male/female)	13/3
Differentiation (well/moderate/poor)	2/11/3
Metastasis (positive/negative)	8/8
TNM stage (I-II/III-IV)	7/9
HBsAg (positive/negative)	14/2
Cirrhosis (positive/negative)	13/3
AFP (positive/negative)	10/6

TNM, tumor-node-metastasis system; HBsAg, hepatitis-B surface antigen; AFP, α-fetoprotein.

expression was quantified using the SYBR PrimeScript RT-PCR kit II (Takara Bio, Inc., Shiga, Japan) and normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method. The PCR primers were as follows: PDCD4, forward, 5'-TGGATGTCCCACATTCATACTCTG-3' and reverse, 5'-TCTGGTTTAAGACGACCTCCATCT-3'; MMP-2, forward, 5'-TGGCGATGGATACCCCTTT-3' and reverse, 5'-TTCTCCCAAGGTCCATAGCTCAT-3'; MMP-9, forward, 5'-TGCCCGGACCAAGGATACAG-3' and reverse, 5'-TCAGGGCGAGGACCATAGAG-3'; c-Jun, forward, 5'-ATC CTGAAACAGAGCATGAC-3' and reverse, 5'-TTGCTGG ACTGGATTATCA-3'; GAPDH, forward, 5'-TCAACGAC CACTTTGTCAAGCTCA-3' and reverse, 5'-GCTGGTGGT CCAGGGGTCTTACT-3'; miR-21, forward, 5'-TAGCTTAT CAGACTGATG-3' and reverse, 5'-TGGTGTCGTGGAG TCG-3'; U6, forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. All qRT-PCR reactions were performed in triplicate.

Western blot analysis. For tissue sample, 100 mg of liquid nitrogen-frozen tissue was ground into powder with a pre-cooled mortar and pestle. Protein extracts of cells or frozen tissue powder were prepared by incubating in a RIPA lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; 1 nM EDTA; 1% leupeptin) (Beyotime). Protein concentration was determined by the BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). A total of 30 μg of proteins were separated on 10% SDS-PAGE, and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk at 37°C for 1 h, membranes were incubated with the appropriate primary antibodies, including anti-PDCD4 (Abcam, Cambridge, UK), anti-c-Jun (Cell Signaling Technology, Irvine, CA, USA), anti-phospho-c-Jun (Cell Signaling Technology) or anti-GAPDH (G9545; Sigma-Aldrich, St. Louis, MO, USA). For reblotting, PVDF membranes were first stripped in a buffer containing glacial acetic acid at 37°C for 60 min, then blocked and probed with a second primary antibody. After washing with Tris buffer, PVDF membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG at 37°C for 1 h. Signals were visualized by using enhance chemiluminescence (GE Healthcare, Waukesha, WI, USA) and X-ray films.
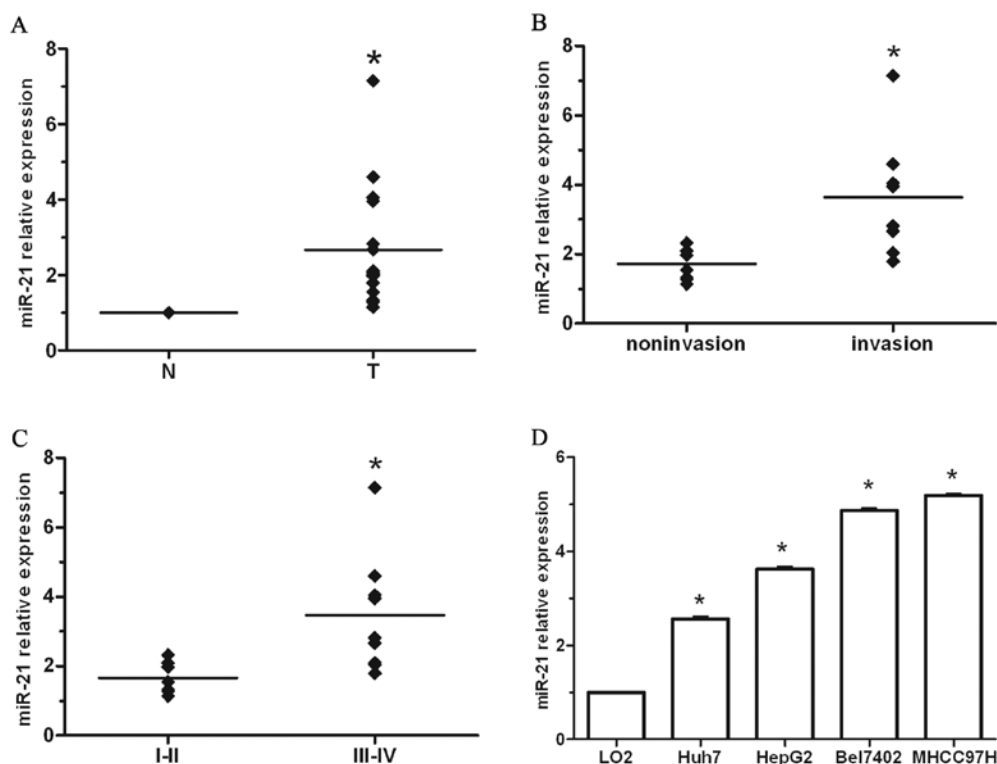
Figure 1. Overexpression of miR-21 was significantly upregulated in HCC tumor tissues/cell lines and was associated with increasing invasive capacity in HCC tissues/cell lines. (A) Relative miR-21 expression levels in tumor tissues and adjacent non-tumor tissues. miR-21 expression was determined in 16 paired HCC tissues by qRT-PCR. The expression levels of miR-21 relative to U6 snRNA were normalized to those detected in the adjacent non-tumor tissue of each matched pair. N, non-tumor tissue; T, tumor tissue. (B) Relative miR-21 expression levels in non-invasive and invasive HCC tumor tissues. (C) Relative miR-21 expression levels in patients with stage III-IV and patients with stage I-II. (D) Relative miR-21 expression levels in HCC cell lines with different invasive capacity (MHCC97H>Bel7402>HepG2>Huh7) and in normal hepatic cells (L02). *P<0.05.

Cell migration and invasion assay. Migration of HepG2 cells was evaluated using transwell chambers with 8 μm pore filters (Corning, Inc., Corning, NY, USA). At 48 or 72 h post-transfection, the miR-21 inhibitor and/or PDCD4 siRNA-transfected cells were trypsinized and $5x10^4$ cells were plated on the upper chamber in serum-free medium. DMEM medium containing 10% FBS was added as a chemoattractant to the lower chamber. After incubating for 12 h at 37°C non-migrating cells were removed with cotton swabs. Cells that migrated to the bottom of the membrane were then fixed with 95% ethanol, stained with 1% eosin for 10 min at 37°C, and washed twice with PBS. Stained cells were visualized under a microscope (high-power field), and the number of cells counted in five random fields were averaged. For the HepG2 invasion assay, $5x10^4$ post-transfected cells were added to the upper chamber that had been pre-coated with 30 μg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Then, DMEM medium containing 10% FBS was added to the lower chamber. Cells were incubated for 24 h at 37°C, and then non-invading cells were removed with cotton swabs. Invaded cells were fixed, stained and counted.

Statistical analysis. Each experiment was performed at least three times. Statistical analysis was performed using the SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA). Values were expressed as mean ± standard error of the mean (SEM). Differences/correlations between the two groups were calculated with the Mann-Whitney U test, Student's t-test, or Pearson's test. Multiple group comparisons were analyzed using ANOVA with a post-test for subsequent individual group comparisons. A P-value of <0.05 was defined as significant.

Results

miR-21 is overexpressed in human HCC tissues/cell lines and is associated with tumor metastasis. To investigate the biological roles of miR-21 in human HCC development, we first examined the expression of miR-21 in 16 paired human HCC tissues and adjacent non-tumor hepatic tissues by miRNA-based qRT-PCR. Consistent with previous reports (16), miR-21 was significantly upregulated in HCC tissues, as compared with that detected in matched non-tumor tissues (Fig. 1A), suggesting that upregulation of miR-21 might be involved in human HCC development.

These 16 HCC tissues were further divided into an invasive/metastatic group (n=8) and a non-invasive/non-metastatic group (n=8), according to intrahepatic metastases, vascular invasion, or capsular invasion. When comparing miR-21 expression between these two groups, we observed significantly higher levels in the invasive/metastatic group (Fig. 1B). Specifically, upregulation of miR-21 was observed in all nine patients with stage III-IV disease, as compared with that in patients with stage I-II disease (Fig. 1C). We next determined whether miR-21 expression was similarly correlated with the invasiveness of HCC cells lines. We compared miR-21 expression in four selected cell lines (MHCC97H, Bel7402, HepG2 and
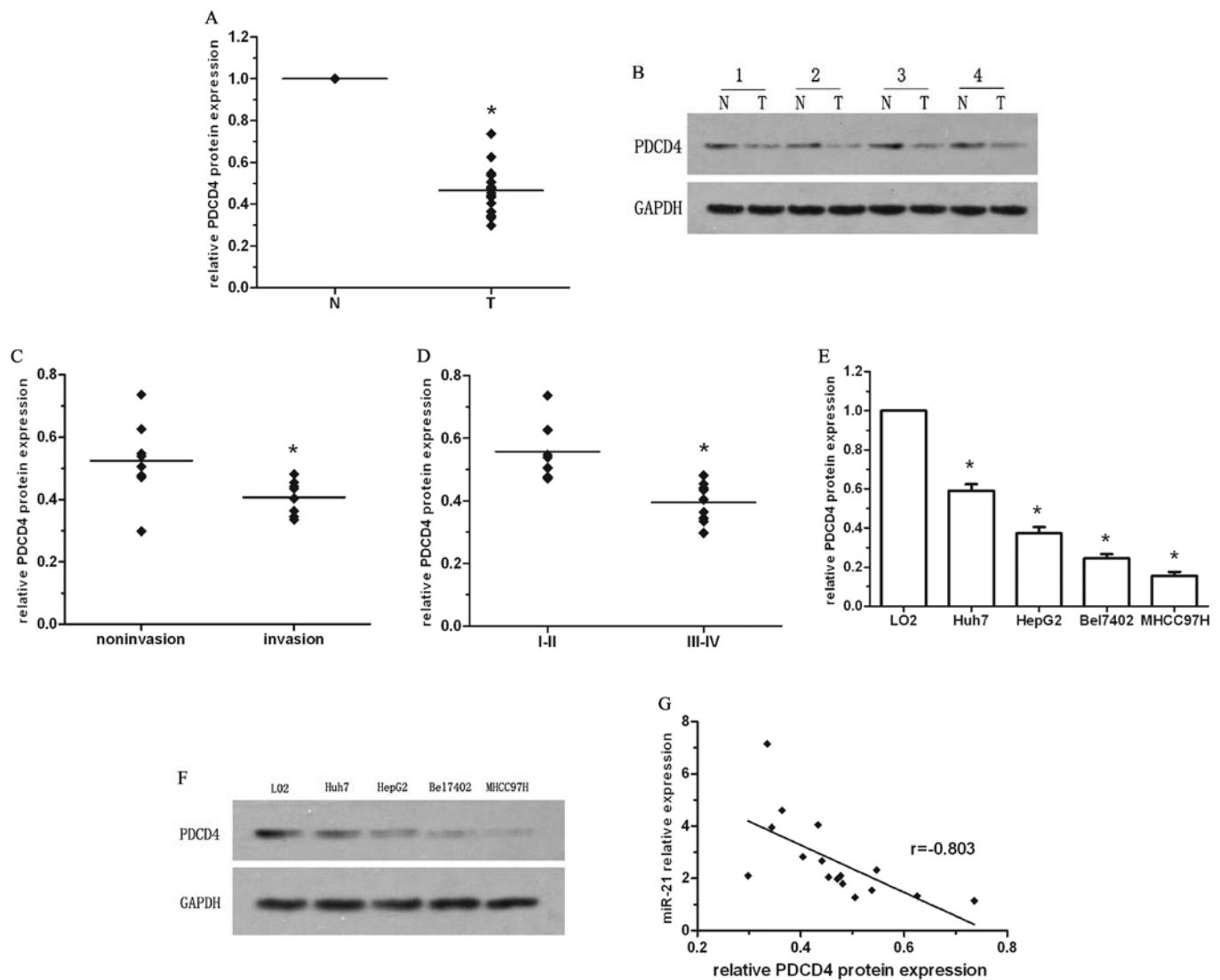
Figure 2. Expression of PDCD4 protein was downregulated in HCC tissues/cell lines with increasing invasive capacity and was inversely correlated to miR-21 levels. (A) Relative PDCD4 protein levels in 16 paired HCC tissues as detected by western blotting. The expression levels of PDCD4 protein relative to GAPDH protein were normalized to the expression of the adjacent non-tumor tissues of each matched pair. N, non-tumor tissue; T, tumor tissue. (B) Representative immunoblot of PDCD4 protein in tumor tissues and adjacent non-tumor tissues. (C). PDCD4 protein was significantly downregulated in invasive/metastatic HCC tissues. (D) PDCD4 protein was significantly downregulated in patients with stage III-IV disease. (E) PDCD4 protein expression was significantly decreased in HCC cell lines, as compared to normal hepatic cells (L02), and was negatively correlated with cell invasiveness. (F) Representative immunoblot of PDCD4 protein in HCC cell lines and normal hepatic cells from three independent experiments. (G) miR-21 expression was inversely correlated with PDCD4 protein in HCC tissues (Pearson's correlation -0.803, P=0.007). *P<0.05.

Huh7) with increasing invasive capacity (22). Indeed, miR-21 was highly upregulated in HCC cell lines, as compared with human normal hepatic L02 cells, and the miR-21 levels were gradually upregulated in the HCC cell lines with increasing invasive capacity (MHCC97H>Bel7402>HepG2>Huh7) (Fig. 1D). Taken together, these data indicated that miR-21 overexpression was significantly associated with the metastasis and invasiveness of HCC.

miR-21 and PDCD4 protein are inversely expressed in human HCC tissues/cell lines. PDCD4 is a previously identified direct target gene of miR-21 and the miR-21-PDCD4 axis has been implicated in the proliferation and apoptosis of pancreatic cancer and glioblastoma cells (19,24). To analyze the clinical significance of miR-21-regulated PDCD4 in HCC, we examined its protein expression in the 16 pairs of matched

HCC specimens by western blotting. In comparison with the non-tumor counterparts, lower levels of PDCD4 were detected in tumor tissues (Fig. 2A), and representative examples are shown in Fig. 2B. Furthermore, PDCD4 protein was downregulated in HCC samples with invasion/metastasis (Fig. 2C) and in patients with stage III-IV disease (Fig. 2D). Among the total 16 pairs of HCC tissues, we observed a significant inverse correlation between PDCD4 protein expression levels and miR-21 levels (P=0.007) (Fig. 2G). In addition, we also analyzed the expression of PDCD4 protein in normal hepatic cells and the above four HCC cell lines. In agreement with the results from tumor tissues, we observed that the levels of PDCD4 in the 4 HCC cell lines were much lower than that in human normal hepatic cells (Fig. 2E and F), and that PDCD4 expression was negatively correlated with the cell invasive capacity and miR-21 (P=0.002) (data not shown). These
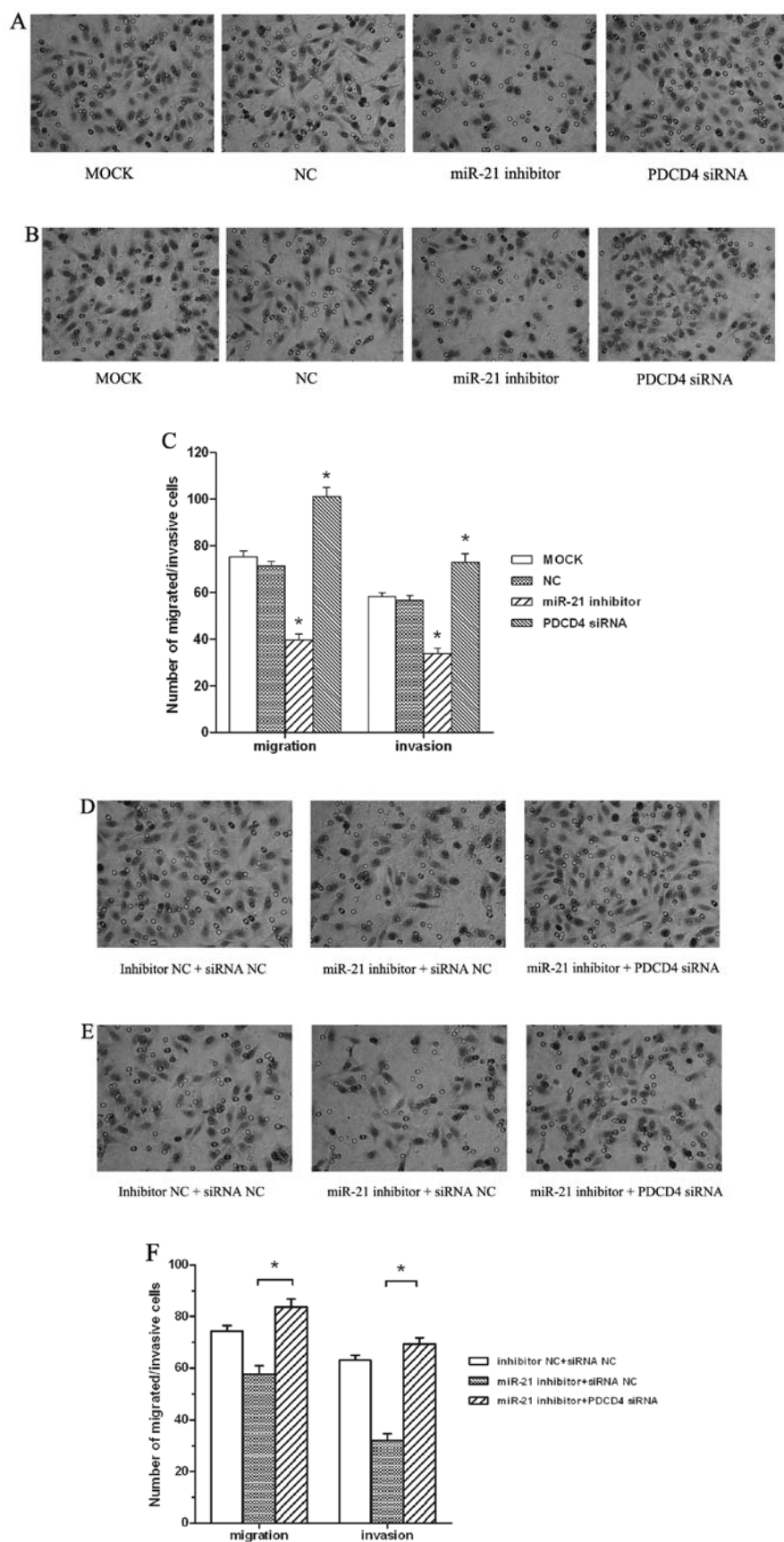
Figure 3. miR-21 promoted migration and invasion in HepG2 cells through PDCD4. (A-C) HepG2 cells were transfected with MOCK, NC, miR-21 inhibitor, and PDCD4 siRNA for 48 h. Transwell cell migration/invasion assays were performed with the cells, as detailed in Materials and methods. Representative images of (A) cell migration and (B) cell invasion from three independent experiments are shown and (C) relative migrated/invaded cells from the above assays were graphed (mean ± SEM). Compared with NC, miR-21 inhibitor transfection repressed migration/invasion of HepG2 cells, whereas PDCD4 siRNA had the inverse effect. (D-F) HepG2 cells were co-transfected with combinations of the miR-21 inhibitor, inhibitor NC, PDCD4 siRNA and siRNA NC. Cell migration and invasion were detected at 72 h post-transfection, as described in the Materials and methods. Representative images of (D) migration and (E) invasion from three independent experiments are shown and relative migrated/invaded cells from (F) the above assays were graphed (mean ± SEM). $^*P<0.05$.
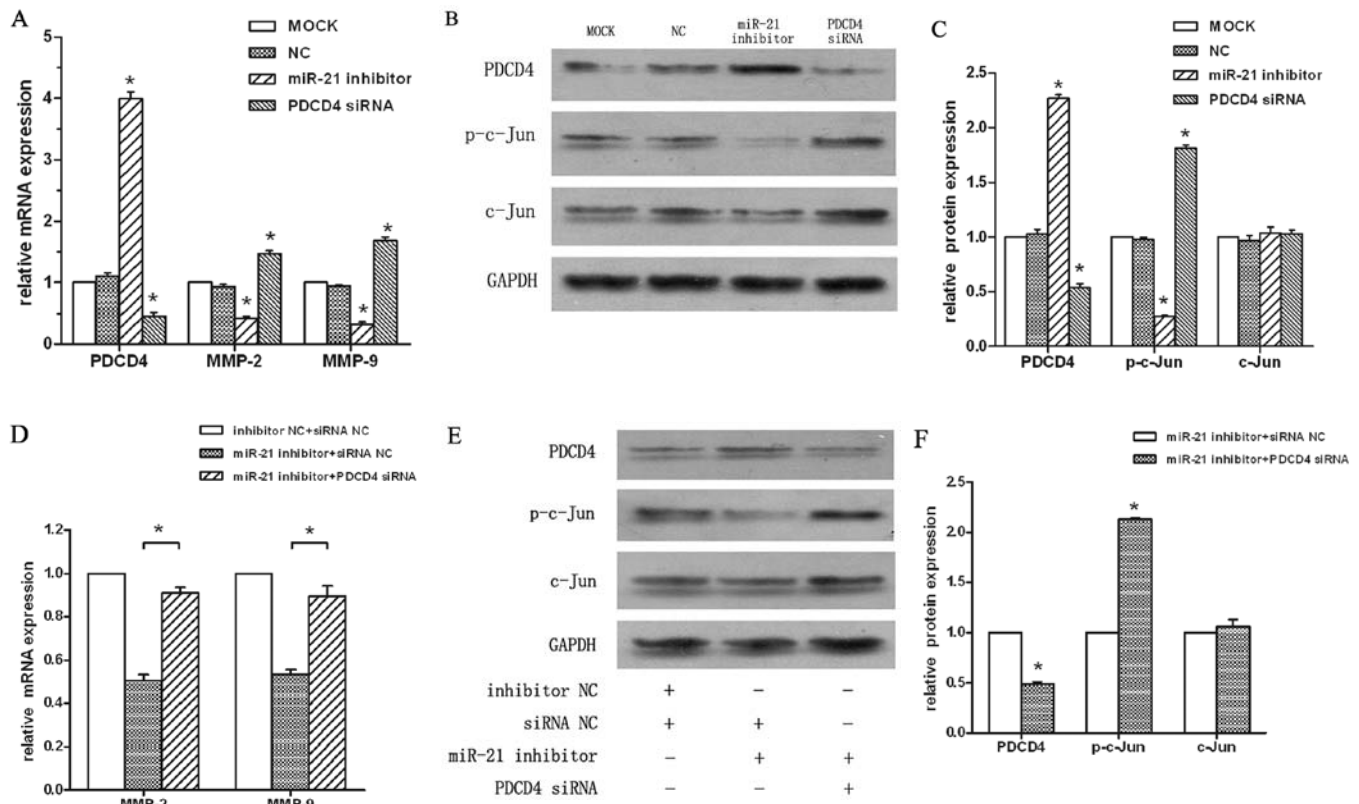
Figure 4. miR-21 suppressed the expression of PDCD4, c-Jun activation, and the MMP downstream signaling molecules. (A-C) HepG2 cells were respectively transfected with MOCK, NC, miR-21 inhibitor or PDCD4 siRNA. (A) After 48 h, total-RNA was isolated and analyzed for PDCD4, MMP-2 and MMP-9 expressions by qRT-PCR. PDCD4, phospho-c-Jun and c-Jun were detected from (B) the same cells by western blotting, and data from (C) three separate experiments were plotted. (D-F) HepG2 cells were co-transfected with combinations of the miR-21 inhibitor, inhibitor NC, PDCD4 siRNA and siRNA NC. (D) The mRNA expression of MMP-2 and MMP-9 were examined by qRT-PCR normalized to that of GAPDH mRNA in the same sample. (E) PDCD4, phospho-c-Jun, and c-Jun levels were evaluated by western blotting, and data from (F) three independent experiments were plotted as mean ± SEM. $^*P<0.05$.
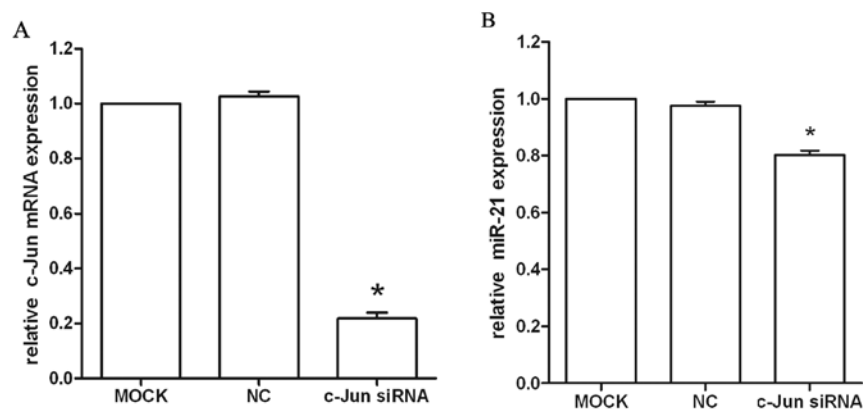

Figure 5. Knockdown of c-Jun inhibited miR-21 expression in HCC cells. HepG2 cells were transfected with MOCK, NC, or c-Jun siRNA for 48 h. (A) c-Jun mRNA and (B) miR-21 expression were detected by qRT-PCR and normalized to GAPDH or U6 snRNA expression, respectively. Data are presented as means of three separate experiments. $^*P<0.05$.

in vitro and *in vivo* data suggest that downregulation of the miR-21 target gene, PDCD4, might also contribute to tumor metastasis/invasion in HCC.

miR-21 regulates migration and invasion of HepG2 cells through targeting PDCD4. Previous studies have found that a miR-21 inhibitor could suppress cell invasion in some malignant cells by promoting translation of specific target genes (8,13,14,16). Therefore, we aimed to determine whether

miR-21 could have the same impact on HCC cells. HepG2 cells were transfected with blank control culture medium (MOCK), inhibitor NC, or miR-21 inhibitor, in order to evaluate cell migration/invasion activity. The miR-21 inhibitor significantly decreased migration/invasion of HepG2 cells, as compared with NC (Fig. 3A-C), indicating that miR-21 positively regulates migration and invasion of cultured HCC cells. Interestingly, knockdown of PDCD4 in HepG2 cells significantly promoted their migration and invasion (Fig. 3A-C).

To further evaluate the contribution of PDCD4 to miR-21-dependent HCC cell migration and invasion, the HepG2 cells were co-transfected with miR-21 inhibitors and PDCD4 siRNAs, and examined for their migration and invasion capacities. The effect of miR-21 inhibition on decreasing both cell migration and invasion was reversed by the presence of PDCD4 siRNA in HepG2 cells, indicating that PDCD4 was required for the miR-21-dependent cell migration and invasion effect in HCC (Fig. 3D-F).

The miR-21 modulates AP-1 signaling in HCC cells by targeting PDCD4. Gene expression is modulated by multiple mechanisms at different levels. To examine whether down-regulation of miR-21 affected the expression of PDCD4 in HCC, HepG2 cells were transiently transfected with the miR-21 inhibitor, NC, or MOCK. The decrease in endogenous miR-21 levels by miR-21 inhibitor significantly increased both PDCD4 mRNA and protein expression levels (Fig. 4A-C). c-Jun, one of the components of transcription factor AP-1, is an important downstream target of PDCD4 and involved in cell apoptosis, proliferation, invasion, and metastasis (29,30). We, thus, compared the levels of total and phosphorylated c-Jun in HepG2 cells following miR-21 inhibition. The expression of phosphorylated c-Jun was decreased by the miR-21 inhibitor. Interestingly, however, total c-Jun protein remained the same (Fig. 4B and C).

Matrix metalloproteinases (MMPs), among which MMP-2 and MMP-9 are the major factors, play an important role in tumor metastasis. PDCD4 suppresses the transcription of several MMPs, including MMP-2 and MMP-9, through down-regulation of AP-1 activation (28). Indeed, mRNA expression of both MMP-2 and MMP-9 was significantly decreased after transfection with miR-21 inhibitor in HepG2 cells, but was further enhanced by PDCD4 knockdown (Fig. 4A), suggesting that miR-21 may exert its biological functions through PDCD4-dependent AP-1 signaling. Therefore, we next investigated the ability of PDCD4 to modulate miR-21-mediated inhibition of MMP-2 and MMP-9 mRNA expression. As expected, miR-21 inhibition-induced downregulation of MMP-2, MMP-9 and phosphorylated c-Jun was reversed by PDCD4 knockdown in HepG2 cells (Fig. 4D-F). Together, these data suggest an important role of PDCD4 as a mediator of the biological effects of miR-21.

AP-1 activates miR-21 transcription. The transcriptional factor AP-1 activates gene transcription by binding to its promoter region. Bioinformatic analysis suggested that AP-1 binding sites are enriched in the putative promoter region of miR-21 (37). To evaluate the regulation of miR-21 expression by c-Jun, HepG2 cells were transfected with c-Jun siRNA and examined for miR-21 expression. miR-21 levels were signifi-cantly downregulated in cells transfected with c-Jun siRNA, as compared to control cells (Fig. 5), suggesting that AP-1 activates miR-21 transcription.

Discussion

As an oncomir, miR-21 is upregulated in a variety of human malignancies, such as cholangiocarcinoma, breast cancer, lung cancer, colorectal cancer, and prostatic carcinoma

(8-12). Consistent with these studies, we demonstrated that miR-21 expression was significantly higher in HCC tissues and cell lines than that in matched non-tumor tissues and normal human hepatic cells, suggesting that high miR-21 expression was tightly associated with tumor development. Inhibition of miR-21 expression could restrain tumor growth in breast cancer cells (9), while overexpression of miR-21 could increase cell invasion in colon carcinoma (12). In this study, we observed that miR-21 expression was dramatically increased in invasive/metastatic HCC tumor tissues and tumor tissues from stage II-IV patients, as compared with that in non-invasive/non-metastatic HCC tumor tissues and tumor tissues from stage I-II patients. We also observed that miR-21 expression was gradually increased in HCC cell lines, along with increased invasive capacity, and inhibition of miR-21 expression could restrain migration and invasion of HepG2 cells. These findings suggest that overexpression of miR-21 was associated with advanced TNM stage and metastasis in HCC.

Tumor suppressor PDCD4 is one of the miR-21 target genes (8). PDCD4 expression is usually reduced or is absent from many human malignancies, including renal-, lung- and glia-derived tumors (23,25). Consistent with these findings, we also observed that the expression of PDCD4 protein in HCC tissues and cell lines was significantly lower than that in matched non-tumor tissues and human normal hepatic cells. In addition, a highly significant negative correlation between miR-21 expres-sion and PDCD4 protein levels in HCC tumor tissues and cell lines existed. Meanwhile, as opposed to miR-21 expression, PDCD4 protein expression was significantly downregulated in metastatic HCC tumor tissues and tumor tissues from stage III-IV patients, and was gradually reduced in HCC cell lines along with increasing invasive capacity. Inhibition of PDCD4 expression could promote migration and invasion of HepG2 cells. Taken together, these data suggest that PDCD4 is a critical mediator through which miR-21 exerts its biological functions in HCC.

Interestingly, a recent study has demonstrated that PDCD4 modulates cancer cell proliferation and invasion by regulating different downstream genes in a cell type/ tissue type-dependent manner. PDCD4 inhibited u-PAR expression and cell invasion and metastasis via SP1/SP3 in colorectal carcinoma cells (17), but in breast cancer cells, PDCD4 over-expression did not affect u-PAR transcription and inhibited cell invasion by increasing TIMP-2 secretion (26). PDCD4 inhibits c-Jun activation and AP-1-dependent transcription via the downstream MAP4K1/JNK/AP-1 signaling pathway in colon carcinoma cells (27,28). AP-1 is a transcription factor complex comprised of Jun-Jun homodimers or Jun-Fos heterodimers (29). The Jun protein family includes c-Jun, JunB and JunD. The Fos protein family contains c-Fos, Fra-1, Fra-2 and FosB. c-Jun is a key molecule in the AP-1 complex. AP-1 regulates multiple biological events required for cell invasion, including expression of the MMPs (30-32) and cell motility (33,34). Overexpression or hyperactivation of AP-1 components enhances invasion (35,36). In the present study, we reported that miR-21 regulated PDCD4 expression at the post-transcriptional and translational levels in HepG2 cells, increased phosphorylation of c-Jun protein without affecting its synthesis, activated AP-1-dependent transcription of

MMP-2 and MMP-9 and promoted migration and invasion of HCC cell lines. Interestingly, we showed that HepG2 cell migration and invasiveness weakened by the miR-21 inhibitor was reversed by knockdown of PDCD4, and that downregulation of the AP-1 component (phospho-c-Jun) and its downstream signaling molecules (MMP-2 and MMP-9) was also rescued by knockdown of PDCD4. These findings supported the hypothesis of PDCD4 playing a critical role in mediating miR-21 dependent biological functions in HCC.

Several conserved enhanced elements have been identified in the consensus sequence of the putative promoter region of miR-21 (miPPR-21) based on TRANSFAC matrices, including binding sites for AP-1, Ets/PU.1, C/EBPα, NFI, SRF, p53 and STAT3 (37). Fujita *et al* (37) reported that AP-1 was capable of activating miR-21 transcription through the conserved AP-1 and PU.1 binding sites in the miPPR-21 after PMA stimulation. In support of the notion that AP-1 could directly activate miR-21 transcription, in this study we showed that miR-21 was downregulated in HepG2 cells transfected with siRNA to c-Jun. Therefore, a positive feedback loop mediated by miR-21-PDCD4-AP-1 could control the AP-1-dependent oncogenes activity, which might be partially involved in invasion and metastasis in HCC. Certainly, additional mechanisms and regulators of the loop are also likely to contribute to miR-21-induced HCC cell invasion, which needs to be further elucidated.

In conclusion, we demonstrated that miR-21 was overexpressed in HCC tissues and cell lines and was a potent stimulator of the migrative/invasive capacities of HCC cells. We further showed that a miR-21 inhibitor restrained HepG2 cell migration and invasion through direct/indirect regulation of PDCD4 and downstream signaling pathway molecules (p-c-Jun/AP-1, MMP-2 and MMP-9), and that the transcription factor AP-1 directly activates miR-21 transcription. We, thus, hypothesize that a positive feedback loop of miR-21-PDCD4-AP-1 maintains the miR-21-mediated biological effects of the HCC malignant phenotype. Our data suggest that molecular introduction strategies interfering with the miR-21-PDCD4-AP-1 feedback loop might provide strong rationale for preventing invasion/metastasis in HCC in the future.

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