

## The miR-93 promotes proliferation by directly targeting PDCD4 in hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the second leading cause of cancer mortality worldwide. Although advances have made in treatment of HCC, the overall survival rate remains low and the molecular pathogenesis of HCC is still poorly understood. The purpose of this study was to explore the molecular pathogenesis of HCC. A total of 89 patients were involved in the study. MicroRNA-93 (miR-93) was aberrantly up-regulated in HCC tissues as determined by qRT-PCR. The high level of miR-93 was closely associated with larger tumor size ( $p < 0.05$ ) and poor overall survival ( $p < 0.05$ ). In *in vitro* and *in vivo* assays, we demonstrated that high miR-93 levels enhanced cell growth of HCC. The luciferase activity assay showed that PDCD4 was a direct target of miR-93 and its expression was down-regulated by miR-93. Re-expression of PDCD4 inversely correlated with the level of miR-93 and attenuated the miR-93-induced promotion of cell growth in HCC. Taken together, our data indicate that miR-93 may function as an oncogenic factor in HCC, and promotes HCC cell proliferation by targeting PDCD4.

*Key words:* growth, hepatocellular carcinoma, miR-93, PDCD4

Hepatocellular carcinoma (HCC) is the second leading cause of cancer mortality worldwide [1]. Hepatitis B and C viral infections are the main environmental causes of HCC. Although advances have made in treatment of HCC, the overall survival rate remains low [2]. Increasing evidence indicates that the aberrant expression of several tumor suppressors or oncogenes such as PTEN, p53 and SIRT1 are involved in HCC tumorigenesis [3], but the molecular pathogenesis of HCC is still poorly understood.

MicroRNAs (miRNAs) are small non-coding RNAs (21–25 nucleotides) that regulates gene expression by binding to the 3'-untranslated region (UTR) of target mRNAs at post-transcription level [4]. Aberrant expression of miRNA leads to multiple diseases, including cancer [5]. To date, several miRNAs have been reported to regulate tumor growth, apoptosis, migration and invasion of HCC, including miR-625 [6], miR-29 [7] and miR-221 [8]. MiR-93 is up-regulated in multiple malignancies, including breast stem cells [9], lung cancer [10] and gastric cancer [11]. These studies show that miR-93 is an oncomiRNA through diverse mechanisms. In HCC, miR-93 is reported to stimulate cell proliferation, migra-

tion and invasion through the c-Met/PI3K/Akt pathway and inhibit apoptosis by directly inhibiting PTEN and CDKN1A [12]. However, the role of miR-93 in the development of HCC remains largely unknown.

Programmed cell death 4 (PDCD4, also known as MA-3, TIS, H731 and DUG) was recently identified as a novel tumor suppressor [13, 14]. Its expression is often decreased in several cancers, including HCC [15]. In HCC, it is demonstrated that PDCD4 is a proapoptotic molecule involved in TGF-beta1-induced apoptosis [15]. Many studies reported that Pdc4 negatively regulates the transactivation of AP-1 and its suppression activates the PI3K/Akt pathway [16]. It showed that PDCD4 could suppress and prevent tumor promotion and progression by regulating various proteins at transcriptional, translational, and posttranslational levels [13]. Thus, we hypothesize that PDCD4 involves in tumor proliferation.

In this study, we provided evidences that miR-93 was aberrantly up-regulated in HCC tissues and correlated to poor overall survival. Over-expression of miR-93 promoted tumor growth significantly both *in vitro* and *in vivo*. We identified that PDCD4 was a functional target of miR-93. The newly

identified miR-93/PDCD4 axis may provide a new potential therapeutic target for HCC treatment.

## Materials and methods

**Cell lines.** Two human HCC cell lines (QGY-7703 and SMMC-7721) were used in the study. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum in a humidified incubator at 37 °C with 5% CO<sub>2</sub>.

**Tissue samples.** A total of 89 patients were involved in the study. Human HCC tissues and their adjacent non-tumorous tissues were collected from patients at the time of surgical resection from Jan 1<sup>st</sup> 2010 to Dec 31<sup>st</sup> 2015 at the Department of Radiology, Airforce General Hospital of PLA, Jinan, Shandong, P. R. China. All cases were histologically confirmed. None of the patients had received radiotherapy or chemotherapy before the surgery. Informed consent was obtained from each patient, and the study was approved by the Institute Research Ethics Committee at the Cancer Center.

**Constructs, oligonucleotides, and transfection.** All RNA oligoribonucleotides were purchased from Genepharma (Shanghai, China). The negative control (NC) RNA duplex for both the miR-93 mimic and the miR-93 inhibitor was non-homologous to any human genome sequences.

A firefly luciferase-expressing vector, pGL3cm vector was used in the luciferase reporter assay. The wild type and mutant type of 3'-UTR segments of PDCD4 were cloned into downstream of firefly luciferase in pGL3cm vector. Mutation of 3'-UTR segments was carried out using Stratagene QuikChange® Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA).

The miR-93 expression vector, pBabe-puro vector was used in the study. The mature miR-93 sequence was amplified by PCR and then inserted into the vector, generating pBpG-miR-93.

The transfection of cells with oligonucleotides was performed by RNAiMAX and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instruction. Co-transfection of miRNA and plasmid DNA was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

**Quantitative real-time RT-PCR.** Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse-transcribed complementary DNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa, Dalian, China), and quantitative RT-PCR (qRT-PCR) was performed with SYBR Premix ExTaq (TaKaRa, Dalian, China). The relative expression ratio was calculated by the 2<sup>-ΔΔCT</sup> method. For miR-93, the expression level was normalized to the endogenous snRNA U6 control. For PDCD4, the expression level was normalized to GAPDH. The primers of PDCD4 were shown as sense: 5'-TATGATGTGGAGGAGGTGGATGTGA-3', antisense: 5'-CCTTTCATCCAAAGGAAAACTACAC-3'. The primers of GAPDH were sense: 5'-GATATTGTTGACATCAATGAC-3', antisense: 5'-TTGATTTGGAGGGATCTCG-3'.

**Western blot assay.** Whole cell extracts were prepared in a lysis buffer. Total protein were separated in 10% SDS-PAGE gels and transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibody for 2 h at room temperature. Protein bands were visualized on Kodak X-ray film using enhanced chemiluminescence ECL substrate (Pierce, Appleton, WI, USA).

**Luciferase reporter assay.** SMMC-7721 cells seeded in 96-well plate were cotransfected with 50 ng of wild type 3'-UTR of PDCD4 or mutant 3'-UTR of PDCD4 and 50 nmol/L miR-93 mimic or miR-NC. Cells were collected 48 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to its corresponding Renilla luciferase activity. All experiments were performed in triplicate and repeated at least in three independent.

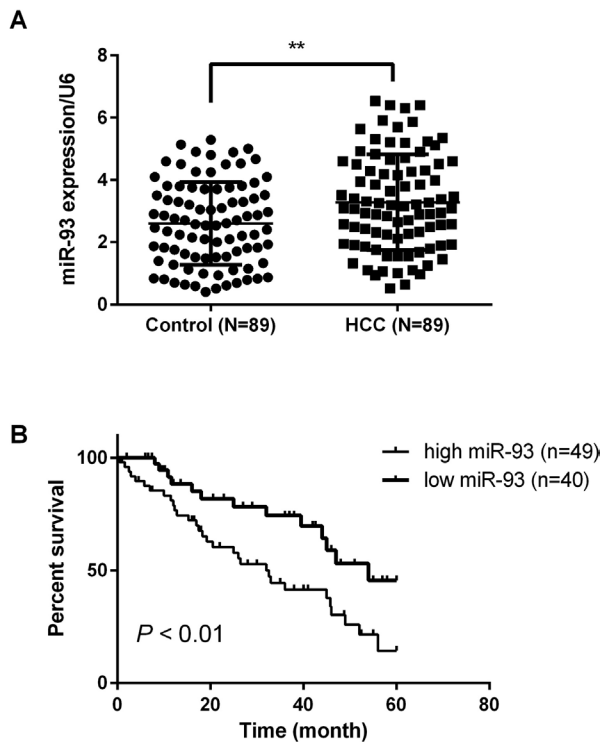
**Cell proliferation assay in vitro.** After transfection, cells were plated in 96-well plates at 3000 cells/well and cultured for 5 days. On the indicated days, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazo-liumbromide reagent (MTT) (AMRESCO, Solon, OH, USA) was added according to the manufacturer's protocol, and the cells were incubated for 4 h at 37 °C. The supernatants were removed, and DMSO (150 μL/well) was added to each well to dissolve formazan crystals. The absorbance at 490 nm for each sample was measured using a multilabel plate reader (PerkinElmer, Waltham, MA, USA).

**In vivo proliferation assay.** For in vivo assays, 10<sup>6</sup> SMMC-7721 cells transfected with miR-93-expressing vector were injected to the flanks of male nude mice. At 6 weeks after injection, the mice were sacrificed and liver tumors were harvested. All animal studies were approved by the Animal Care and Use Committee of Jinan Infectious Disease Hospital.

**Statistical analysis.** Results were presented as means ± standard deviation (SD). Comparisons between groups were detected using Student's t-test. The analysis of miR-93 in the prediction of patient's survival was performed using Kaplan-Meier method. Differences were considered significant for P-values less than 0.05.

## Results

**MiR-93 is aberrantly up-regulated in HCC tissues and associated with poor overall survival.** A total of 89 pairs of HCC tissues and adjacent non-tumorous tissues were involved in the study. To determine the expression of miR-93 in HCC tissues, qRT-PCR analysis was performed. The results showed that the expression of miR-93 in HCC tissues was significantly up-regulated than that in control tissues (Figure 1A). We further analyzed the clinicopathological characteristics of the 89 patients. It showed that the high level of miR-93 was closely associated with larger tumor size ( $p < 0.01$ ). However, no significance was observed with respect to gender, age, re-



**Figure 1.** The miR-93 is generally up-regulated in HCC tissues. (A) The expression of miR-93 in HCC tissues was significantly higher than that in control tissues ( $p < 0.05$ ). (B) Kaplan-Meier survival analysis showed that the patients with higher miR-93 expression had a significant poorer 5-year overall survival ( $p < 0.05$ )

**Table 1.** Clinicopathological characteristics analysis of 89 hepatocellular carcinoma patients

Characteristics	Relative expression of miR-93		P-values*
	Low (n=40)	High (n=49)	
Age (year)			
≤ 45	15 (40.5%)	22 (59.5%)	0.523
> 45	25 (48.1%)	27 (51.9%)	
Gender			
Male	26 (46.4%)	30 (53.6%)	0.826
Female	14 (42.4%)	19 (57.6%)	
Tumor differentiation			
I+II	18 (47.4%)	20 (52.6%)	0.830
III+IV	22 (43.1%)	29 (56.9%)	
Relapse			
Yes	19 (46.3%)	22 (53.7%)	0.834
No	21 (43.8%)	27 (56.2%)	
Tumor size (cm)			
< 5	28 (58.3%)	20 (41.7%)	<b>0.0099</b>
≥ 5	12 (29.3%)	29 (70.7%)	
AFP (ng/mL)			
< 20	15 (46.9%)	17 (53.1%)	0.827
≥ 20	25 (43.9%)	32 (56.1%)	

\* $\chi^2$  test.

lapse and AFP (Table 1). The Kaplan-Meier survival analysis revealed that the patients with higher miR-93 expression had a poor overall survival prognosis compared with patients with lower miR-93 expression (Figure 1B). These results indicated that miR-93 was up-regulated in HCC cells and associated with poor prognosis.

**Over-expression of miR-93 enhances HCC cell proliferation in vitro.** To evaluate the functional role of miR-93 in HCC cells, we re-expressed miR-93 and inhibited miR-93 expression in both QGY-7703 cells and SMMC-7721 cells to measure cell proliferation ability using MTT method. The successful over-expression of miR-93 and knockdown of miR-93 were determined by qRT-PCR. MTT assay showed that cell proliferation was significantly increased in miR-93-over-expression cells compared with control cells (Figure 2A), and miR-93 inhibitor significantly reduced cell proliferation in two cells (Figure 2B).

**MiR-93 down-regulates PDCD4 by directly targeting its 3'-UTR.** To explore downstream targets of miR-93, we conducted a bioinformatics analysis to predict miR-93 targets using the three websites, TargetScan ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)), PicTar (<https://omictools.com/pictar-tool>) and miRanda (<http://www.mirbase.org/>). PDCD4 was predicted as a putative downstream target of miR-93 (Figure 3A). To confirm the prediction, a luciferase reporter assay was performed in SMMC-7721 cells. The luciferase activity of PDCD4 3'-UTR was remarkably inhibited in response to the transfection of miR-93 mimics. The effect was abolished when mutant type of 3'-UTRs and miR-93 were co-transfected into cells (Figure 3B). In addition, the over-expression of miR-93 significantly reduced the endogenous PDCD4 expression at protein level in SMMC-7721 cells, but PDCD4 mRNA levels were not significantly decrease compared with control (Figure 3C). The knockdown of miR-93 in SMMC-7721 cells did not significantly reduced PDCD4 mRNA levels but increased the expression of PDCD4 at protein level (Figure 3D). These results indicate that miR-93 down-regulates PDCD4 expression in a post-transcriptional mechanism by directly targeting its 3'-UTR.

**PDCD4 reverses the miR-93-induced promotion of cell growth in vitro.** Previous study showed that miR-93 can directly down-regulate PDCD4 expression. To further validate whether over-expression of PDCD4 inversely correlates with miR-93-induced promotion, plasmids expressing PDCD4 without its 3'-UTR were constructed. Besides, siRNA was transfected into SMMC-7721 cells to inhibit PDCD4 expression. We infected SMMC-7721 cells with a control vector or miR-93 mimic or miR-93 mimic plus PDCD4 plasmids or PDCD4 plasmids. The expression levels of PDCD4 in SMMC-7721 cells were confirmed by western blot (Figure 4A). The cell growth ability was assessed using MTT method. The over-expression of PDCD4 reduced cell proliferation compared with control cells, and dramatically reversed the promotion of cell proliferation induced by miR-93, and the knockdown of PDCD4 significantly increased cell proliferation (Figure 4B).

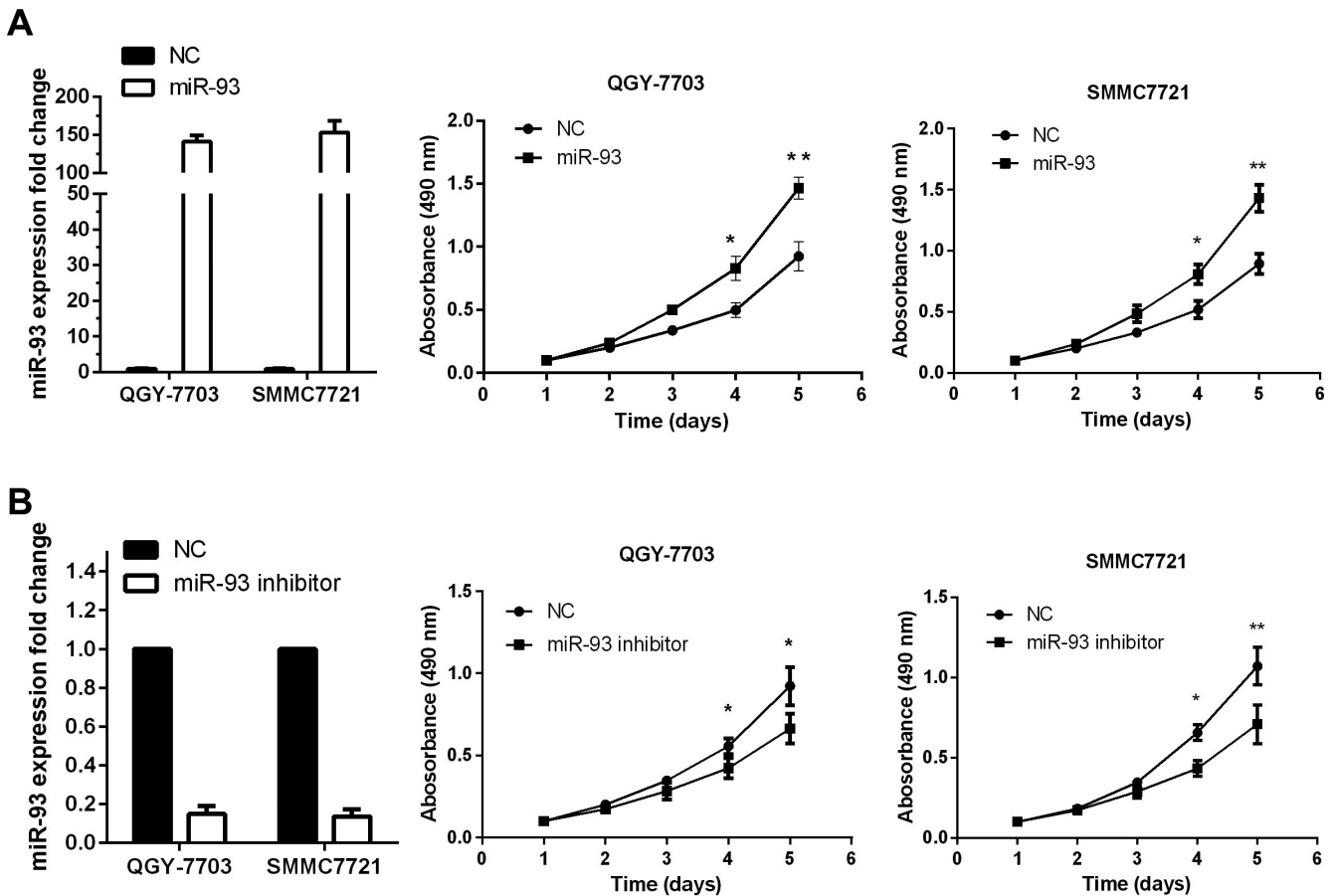


Figure 2. Re-expression of miR-93 promotes HCC cell growth measured by MTT *in vitro*. (A) The successfully re-expression of miR-93 was detected by qRT-PCR. (B) MTT assays showed that the re-expression of miR-93 dramatically increased cell proliferative ability.

**The re-expression of miR-93 promotes HCC proliferation *in vivo*.** *In vitro* assays, miR-93 showed a promotion effect on HCC cell growth. To investigate whether miR-93 promotes tumor proliferation *in vivo*, SMMC-7721 stably expressing miR-93 were injected into the flanks of node mice. After 6 weeks of inoculation, the tumor size was measured. In the miR-93 group, tumor size was significantly bigger than in control group, as shown in Figure 5. Thus, these data suggests that miR-93 may promote tumor growth *in vivo*.

## Discussion

In the miRNA profiling, numerous miRNAs were revealed aberrantly expressed, including miR-93, which was up-regulated in HCC tissues [17]. However, the biological function of miR-93 in HCC was poorly understood. In this study, we revealed that miR-93 expression levels were increased in HCC tissues and was significantly associated with poor overall survival. In *in vitro* and *in vivo* assays, we demonstrated that the miR-93 had a promotion effect on cell proliferation of HCC. We also identified the PDCD4 as

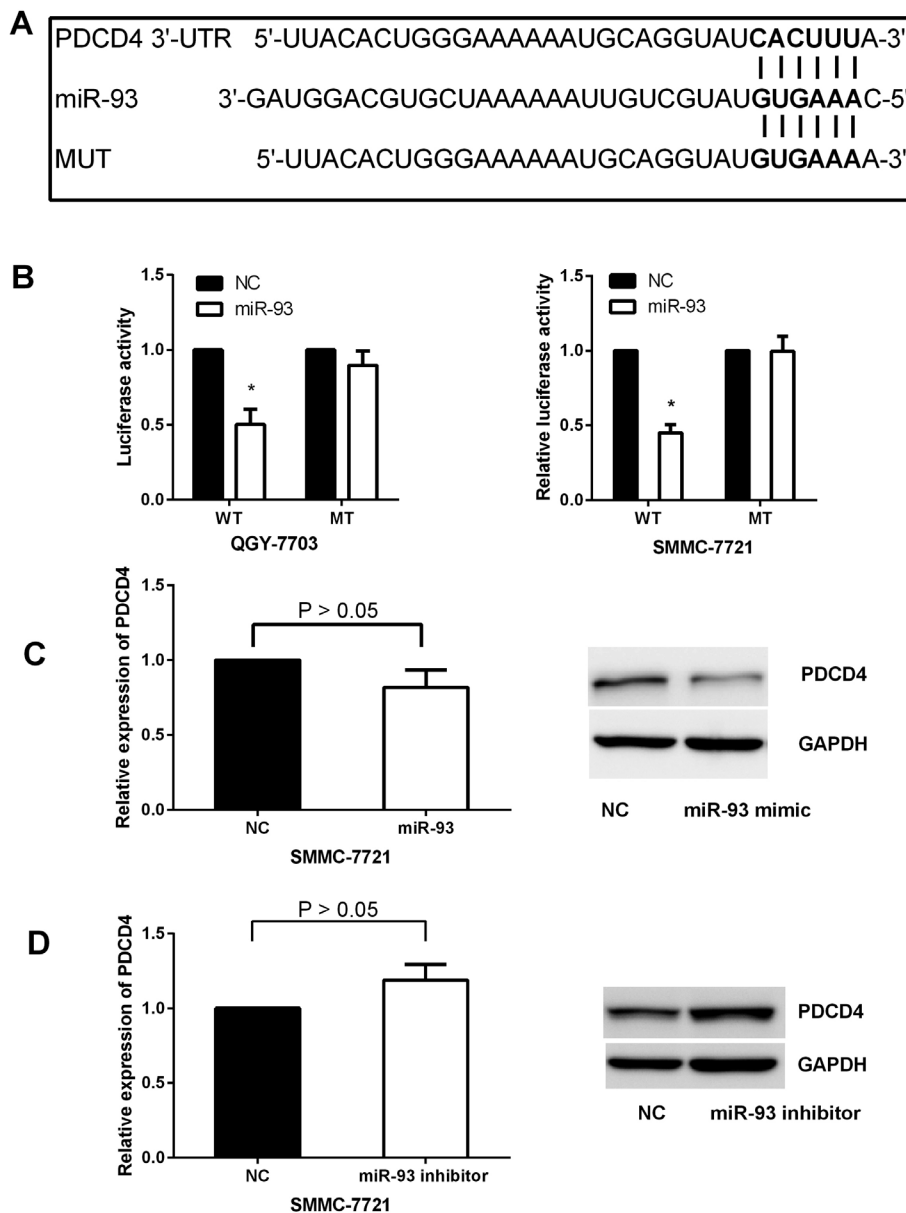
a functional and direct target of miR-93, and its suppressive role on HCC proliferation was facilitated by reversing the miR-93-induced promotion of cell growth. These evidences indicate that up-regulation of miR-93 plays an important role in the process of HCC growth and miR-93 may be potential therapeutic target for HCC treatment.

Various studies showed that dysregulation of miR-93 was associated with a lot of human cancers. High levels of miR-93 inhibited Fus1 and DAB2 expression in lung cancers, which are tumor suppressor genes [10, 18]. MiR-93 is identified promoting ovarian granulosa cells proliferation by targeting CDKN1A in polycystic ovarian syndrome [19]. In gliomas, miR-93 promotes cell proliferation through activation of PI3K/Akt signaling pathway [20]. In nasopharyngeal carcinoma, microRNA-93 promotes cell growth and invasion through targeting disabled homolog-2 [21]. A recent study demonstrated that miR-93 is up-regulated in HCC and stimulated cell proliferation, migration and invasion, and additionally inhibited apoptosis *in vitro* by activating c-Met/PI3K/Akt pathway activity by directly inhibiting PTEN and CDKN1A [12]. These data suggest

that miR-93 play a key role in tumor progression. While in this study, we revealed that miR-93 was significantly up-regulated and was significantly correlated with tumor size. In vitro MTT assay and in vivo assay showed that high levels of miR-93 enhanced cell growth in vitro and in vivo. Both clinical and experimental data suggested miR-93 as an oncogenic factor in HCC.

The fundamental function of miRNAs is to regulate their target genes by inhibition of protein synthesis and/or by

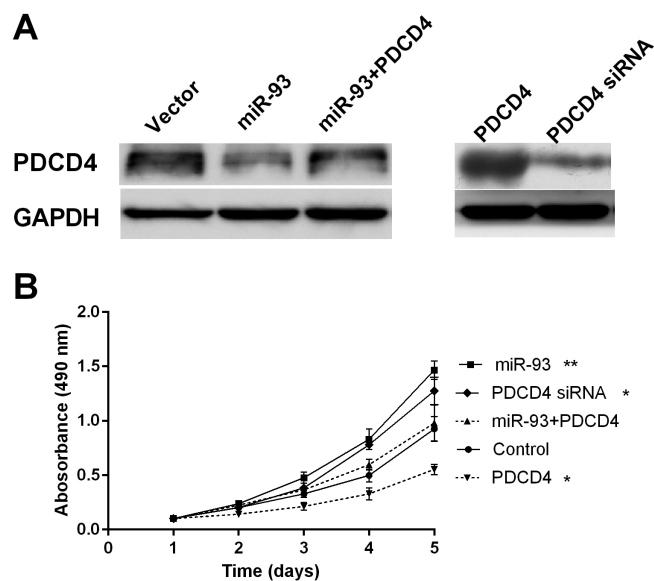
direct cleavage of the mRNA, according to the degree of complementarity with the target mRNA 3'-UTR [22]. Our present study revealed that miR-93 inhibits PDCD4 expression. Bioinformatics prediction provides the putative binding site of miR-93 containing a putative binding site within the 3'-UTR region. The prediction was validated by luciferase activity reporter assay. We observed that over-expression of miR-93 reduced the PDCD4 expression and the over-expression of PDCD4 expression successfully attenuates the



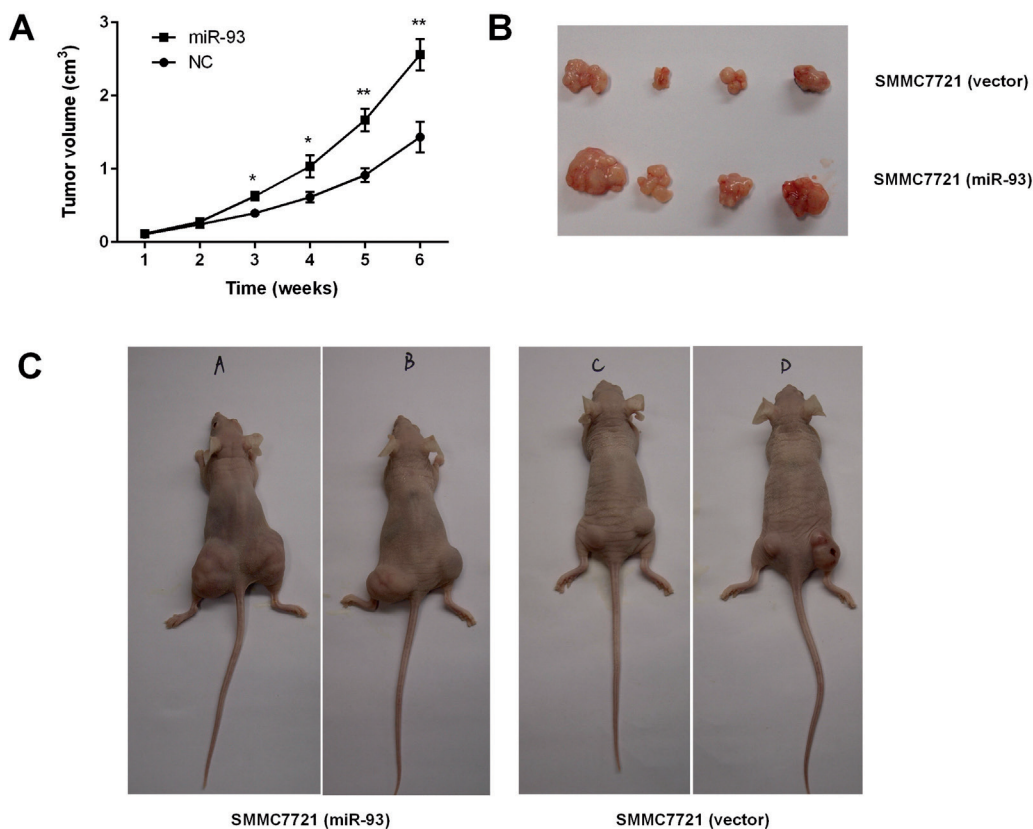
**Figure 3.** The miR-93 down-regulates PDCD4 expression by directly targeting its 3'-UTR. (A) The putative binding site in the 3'-UTR of PDCD4. (B) Luciferase reporter assays showed that reporter activity was decreased after cells were co-transfected with miR-93 and mutation of 3'-UTR of PDCD4. (C) PDCD4 expression was down-regulated by re-expression of miR-93 as determined by western blot. (D) PDCD4 expression at protein level was up-regulated by silencing miR-93 expression.

promotion effects of miR-93 on cancer cell growth. These results suggest that by targeting PDCD4 is a major mechanism miR-93 functions a tumor-promotional role. PDCD4 is a tumor suppressor protein that interacts with eukaryotic initiation factor 4A and inhibits protein synthesis [23]. It is reported that PDCD4 is a target of Akt by phosphorylating PDCD4 in a PI3K-dependent manner, resulting in nuclear translocation of PDCD4 [24]. In earlier study, PTEN and CDKN1A were also identified as targets of miR-93 [12]. PTEN is a tumor suppressor that inhibits phosphorylation of Akt by dephosphorylating PIP3 back to PIP2 [25]. CDKN1A is a potent cyclin-dependent kinase inhibitor and functions as a regulator of cell cycle progression at G1 and S phase [26]. Growth arrest by p21 can promote cellular differentiation, therefore preventing cell proliferation [27]. Thus PDCD4 showed different inhibition mechanism with PTEN or CDKN1A.

Taken together, we provide evidence that miR-93 promotes tumor cell growth of HCC in vitro and in vivo. PDCD4 is identified as a direct and functional target of miR-93 and its expression is reduced by miR-93 at protein levels. The identified miR-93/PDCD4 axis would help to better understand the molecular mechanisms underlying HCC development and provide potential therapeutic targets for HCC treatment.



**Figure 4.** Re-expression of PDCD4 reverses the miR-93-induced promotion of cell proliferation in vitro. (A) Re-expression of PDCD4 was determined by western blot. (B) Compared with control cells, re-expression of PDCD4 significantly reduced cell proliferation ( $P < 0.05$ ), miR-93 significantly increased cell proliferation ( $P < 0.01$ ) and PDCD4 siRNA significantly increased cell proliferation ( $P < 0.05$ ). \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 5.** The re-expression of miR-93 enhances HCC growth in vivo. (A) Tumor growth curves were measured every 7 days. (B) and (C) Tumor volume at 35 days after implantation.

Still, there are limitations in the study. The samples of patients were small and only two cell lines were used in the study. The gene function was not fully understood. We will perform a systematic research in the further study.

## Conclusions

Our data indicate that miR-93 may function as an oncogenic factor in HCC, and the miR-93/PDCD4 axis may present a new potential therapeutic target for HCC treatment.

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