

MiR-877-5p suppresses cell growth, migration and invasion by targeting cyclin dependent kinase 14 and predicts prognosis in hepatocellular carcinoma

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Abstract. – **OBJECTIVE:** Recent studies reveal that hepatocellular carcinoma (HCC) express aberrant microRNAs. Dysregulation of miR-877-5p has been observed in HCC. The objective of the present study was to explore the clinical significance, function and underlying mechanism of miR-877-5p in HCC.

PATIENTS AND METHODS: Quantitative reverse transcription PCR (qRT-PCR) was used to measure the levels of miR-877-5p in HCC specimens and HCC cell lines. Correlations between miR-877-5p expression and the clinicopathological features and prognosis of HCC patients were then evaluated. MTT assays, colony formation assays, scratch test, transwell assays were used to explore the biological function of miR-877-5p in HCC. A luciferase reporter assay and Western blot were conducted to confirm the target gene of miR-877-5p, and the results were validated in HCC cell lines.

RESULTS: We found that the expression of miR-877-5p was downregulated in HCC tissues or cell lines. Clinicopathologic analysis revealed that low miR-877-5p expression correlated with histologic grade ($p = 0.008$) and TNM stage ($p = 0.018$). The Kaplan-Meier method indicated that low miR-877-5p levels in HCC were associated with shorter overall survival ($p = 0.0041$) and disease-free survival ($p = 0.0005$). Multivariate analysis demonstrated that miR-877-5p expression was an independent poor prognostic factor for HCC patients. Functional assay revealed that upregulation of miR-877-5p could inhibit proliferation, migration, and invasion of HCC cells *in vitro*. We further identified cyclin-dependent kinase 14 (CDK14) as a direct target of miR-877-5p in HCC cells. Ectopic expression of CDK14 reversed the inhibitory effects of miR-877-5p.

CONCLUSIONS: Low miR-877-5p expression was a poor prognostic factor for HCC patients, and miR-877-5p functioned as a tumor suppressor in HCC cells via targeting CDK14.

Key Words:

MiR-877-5p, Hepatocellular carcinoma, CDK14, Invasion, Migration, Prognosis.

Introduction

Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related mortality in men, with an increasing incidence in recent years¹. Although the surgical techniques and instruments for HCC surgery have been improved dramatically in recent year, the prognosis of patients with HCC remains extremely poor, which is caused by the high occurrences of metastases and postsurgical recurrence^{2,3}. Therefore, it is of critical importance for us to elucidate potential mechanisms correlated to HCC metastasis.

MicroRNAs (miRNAs) belong to a class of short, highly conserved non-coding RNAs that can negatively regulate gene expression by binding to the 3'untranslated region (UTR) of the target mRNAs, leading to their degradation or repression of mRNA translation^{4,5}. It has been reported that miRNAs play a role in diverse cell, physiological and pathophysiological processes⁶. Importantly, growing studies^{7,8} have indicated that aberrant expression of miRNAs is closely associated to proliferation, apoptosis, metastasis

and pathogenesis of various cancers. In addition, the function and mechanism of a number of miRNAs in HCC have been identified previously. For instance, miR-379-5p was down-regulated in HCC tissues and targets FAK/AKT signaling and inhibits proliferation and metastasis of HCC cells⁹. MiR-1180 was significantly highly expressed in HCC and its knockdown suppressed the proliferation of HCC cells by modulating TNIP2 expression¹⁰. Those findings highlighted miRNAs as potential biomarkers involved in metastasis, suggesting that miRNA could be used as novel therapeutic targets. However, the function and mechanism of miRNAs in tumorigenesis and development remain largely unclear.

MiR-877-5p, which maps to chromosomal region 6, has been reported to be dysregulated in several diseases, including cancers¹¹⁻¹³. It was found that the expression levels of miR-877-5p served as a tumor suppressor in several tumors^{14,15}. Although previous work¹⁶ found that miR-877-5p was highly expressed in HCC and suppressed HCC proliferation by targeting FOXM1, the prognostic value of miR-877-5p in HCC patients has not been reported. At the same time, the potential mechanism underlying metastasis needed to be further studied.

Patients and Methods

Patients and Tissue Samples

147 paired human HCC and adjacent non-tumorous tissues were collected from the Jianhu

People's Hospital. All patients were diagnosed as HCC according to histological examination. None of the patients received chemotherapy or radiotherapy prior to surgery. The survivals of these patients were followed up with a median period of 60 months. Overall survival (OS) time was calculated from the date of the initial surgery to death. Disease-free survival (DFS) was defined as time from diagnosis to disease recurrence or death. The clinicopathological features all the patients were indicated in Table I. Written informed consents were obtained from all patients, and the protocol was approved by the Ethical Committee of the Jianhu People's Hospital.

Cell Culture and Transfection

Four HCC cell lines (HepG2, Huh7, Hep3B, and Bel-7402), and the human normal hepatocyte cell line (LO2) were obtained from the Cell Resource Center of the Chinese Academy of Medical Sciences (Haidian, Beijing, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Haidian, Beijing, China) as well as 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified environment containing 5% CO₂.

MiR-877-5p mimics and its negative control were chemically synthesized by Shanghai GenePharma Co., Ltd. (Pudong, Shanghai, China). The pcDNA/CDK14 and Control were purchased from Life Technologies (Thermo Fisher Scientific).

Table I. Correlations between miR-877-5p and clinicopathological variables of HCC.

Parameters	Group	Total	miR-877-5p expression		p value
			High	Low	
Gender	Male	92	42	50	0.297
	Female	55	30	25	
Age (years)	< 60	64	33	31	0.582
	≥ 60	83	39	44	
Tumor size	< 5	88	47	41	0.190
	≥ 5	59	25	34	
Tumor number	Solitary	56	30	26	0.382
	Multiple	91	42	49	
AFP	< 20	52	24	28	0.612
	> 20	95	48	47	
Hepatitis B	Negative	41	20	21	0.976
	Positive	106	52	54	
Histologic grade	High	86	50	36	0.008
	Low	61	22	39	
TNM stage	I-II	92	52	40	0.018
	III-IV	55	20	35	

ic, Waltham, MA, USA). HepG2 and Huh7 cells were seeded in six-well plates for 24 h and then transiently transfected with miR-877-5p mimics, pcDNA/CDK14, or their controls using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). QRT-PCR assays were performed to determine the transfection efficiency.

Reverse Transcription and Real-time PCR

Total-RNA from tissues and cell lines was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Complementary DNA synthesis was performed using the Prime Script RT Reagent Kit (TaKaRa, Dalian, China). Real-time PCR was performed using FastStart Universal SYBR Green Master kit (Roche Diagnostics, Pudong, Shanghai, China) and analyzed with an Applied Biosystems 7900 Real-time PCR System. GAPDH was used as an internal control. All primer sequences are listed in Table II. Data were collected and analyzed using the $2^{-\Delta\Delta C_t}$ method for quantification of the relative mRNA expression levels.

MTT Assay

Cell growth was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, the transfected cells were plated in 96-well plates (3000 cells per well) 24 h after transfection and cultured at 37°C and 5% CO₂ atmosphere. Then, cells were washed two times with PBS and MTT solution (5 mg/ml) was added to each well. Cell viability was assessed at an absorbance of 490 nm.

Colony Formation Assay

Transfected HepG2 and Huh7 cells (500 cells/well) were seeded in 6-well plates and maintained in media containing 10% FBS, replacing the medium every 4 d. After 7 days, cells were washed twice with phosphate-buffered saline (PBS), fixed

with formaldehyde for 30 min, stained with crystal violet. Colonies containing at least 50 cells were counted to evaluate the colony formation ability.

Scratch Migration Assay

For wound-healing migration assay, cells were seeded on 12-well plates at a density of 1×10^5 cells/well in the culture medium. A scratch was generated by use of a sterile 10-μl pipette tip, and this time was considered as 0 h. Images were captured at the same site at 0, 24 after scratching to observe the process of wound healing.

Transwell Assay

1×10^5 cells were plated in the upper chamber precoated with Matrigel (Biosystems, Foster City, CA, USA) for the invasion assay. Medium containing 20% FBS was placed in the lower chamber. After the non-invading cells were removed from the upper surface of the membrane with a cotton swab, invaded cells on the lower membrane surface were fixed with methanol and glacial acetic acid and then stained with 20% Giemsa solution. For quantification, cells were counted under a microscope in five fields.

Luciferase Reporter Assay

The potential miR-877-5p-binding sites in CDK14 3'UTR were predicted by TargetScan and miRanda. For pGL3-CDK14-3'-UTR-Mut vector (Mt vector), the predicted binding region of miR-877-5p was mutated. HepG2 and Huh7 cells were seeded in 6-well plates and transfected with plasmid and miR-877-5p mimics when cells reached 70% confluence. Cells were harvested 48 h after transfection and the luciferase activities were assayed. The tests were repeated in three independent experiments.

Western Blot

Cell protein concentration was identified by Bio-Rad DC protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts of proteins were separated by dodecyl sulfate, sodium salt-Polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride (PVDF) membranes (Biosystems, Foster City, CA, USA). Membranes were blocked with 5% non-fat milk and then incubated overnight with the appropriate primary antibody. The next day, blots were washed with PBS and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. Protein detection was performed using an ECL chemiluminescence reagent.

Table II. The primers for RT-PCR.

Gene	Primer sequence (5'-3')
miR-877-5p Fwd	GTAAGAGAGATGGCGCAGGG
miR-877-5p Rev	CAGTGCGTGTCGTGGAGT
CDK14 Fwd	TGGGAAGTTGGTAGCTCTGAA
CDK14 Rev	CCAGGGTGCTTGTCCATGTA
GAPDH Fwd	CATCTTCCAGGAGCGAGA
GAPDH Rev	TGTTGTCATACTTCTCA

The primary antibodies used were: anti-GAPDH, anti-CDK14 (Epitomics, Burlingame, CA, USA). Intensities of the bands were normalized to the corresponding GAPDH bands.

Statistical Analysis

Statistical analysis and data plotting were conducted with GraphPad Prism, version 5 (GraphPad Software, Inc., La Jolla, CA, USA). The differences between groups were analyzed using Student's *t*-test. Multiple comparison between the groups was performed using Student-Newman-Keuls test method. Relationships between miR-877-5p expression level and the clinicopathological characteristics were studied using the χ^2 -test. Survival analyses were performed using the Kaplan-Meier method and the log-rank test. The significance of survival

variables was evaluated using a multivariate Cox proportional hazards regression analysis. A *p*-value < 0.05 were considered statistically significant.

Results

Expression Level of miR-877-5p in HCC Tissues and its Relationship with Clinicopathological Features

MiR-877-5p expression levels in 147 pairs of HCC tissues and pair-matched adjacent normal tissues were examined by qRT-PCR. We observed that the relative levels of miR-877-5p in HCC specimens were remarkably lower than those in adjacent noncancerous hepatic tissues (*p* < 0.01) (Figure 1A). Moreover, the expression

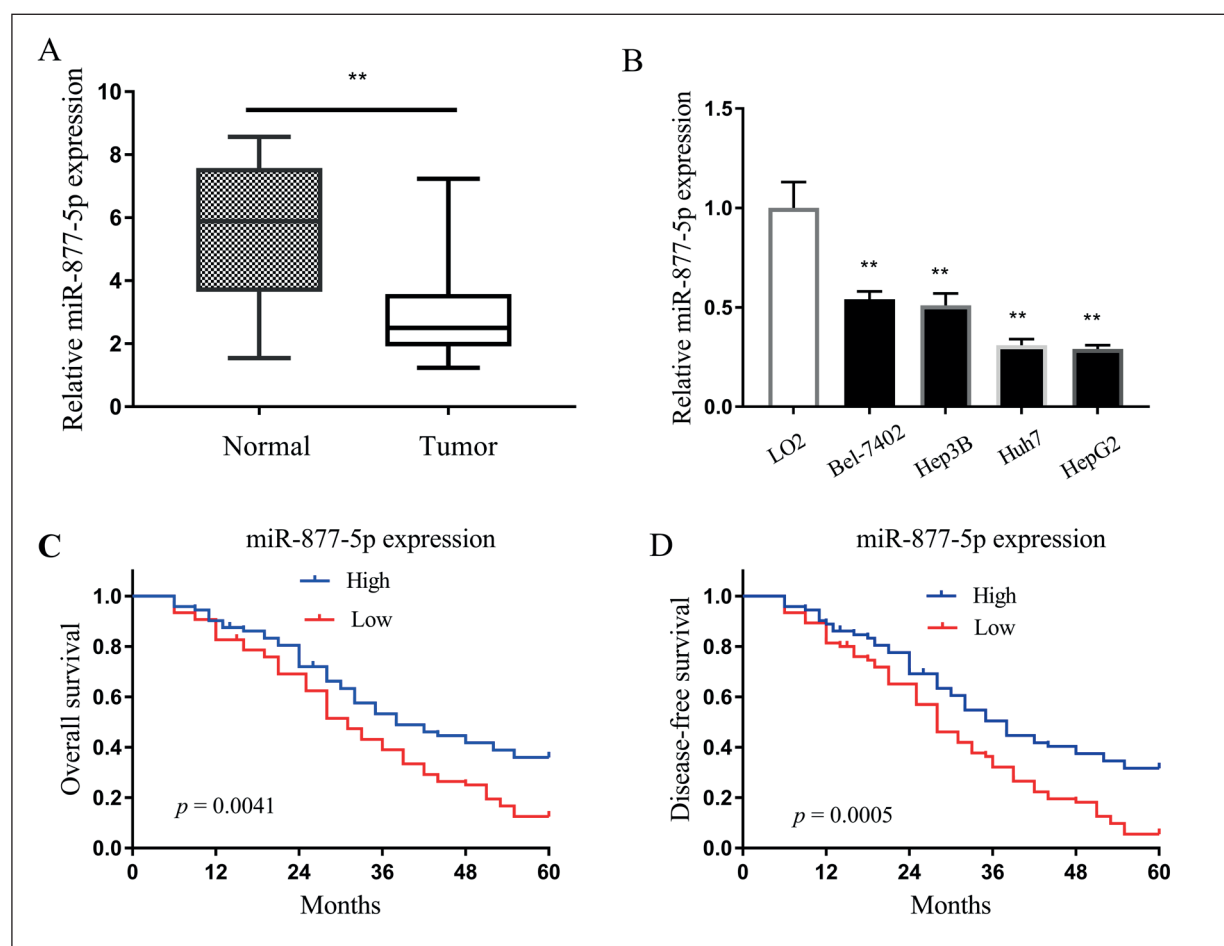


Figure 1. Down-regulation of miR-877-5p in HCC tissues and cell lines was detected by qRT-PCR. (A) RT-PCR was performed to detect the expression of miR-877-5p in HCC tissues and matched normal tissues. (B) Comparing differences in the expression levels of miR-877-5p between HCC cell lines and the non-transformed hepatic cell line LO2. (C) Kaplan-Meier survival analysis of overall survival in 147 HCC patients according to miR-877-5p expression. (D) Kaplan-Meier survival analysis of disease free survival in 147 HCC patients according to miR-877-5p expression. **p* < 0.05, ***p* < 0.01.

of miR-877-5p was also detected in the HCC cell lines. RT-qPCR showed that miR-877-5p expression in four HCC cell lines was remarkable downregulated compared to LO2 (Figure 1B). Then, we divided the HCC patients into a high expression group ($n = 72$) and a low expression group ($n = 75$), according to the median expression level of miR-877-5p. Table I summarized the association between miR-877-5p expression and clinicopathologic features in HCC. It was observed that high expression of miR-877-5p was positively associated with histologic grade ($p = 0.008$) and TNM stage ($p = 0.018$) in HCC patients. Our results suggested that miR-877-5p may play an important role in progression of HCC.

Prognostic of miR-877-5p in HCC Patients

In order to further explore the value of miR-877-5p in outcome of patients with HCC, we used Kaplan-Meier survival analysis and log-rank tests. We found that OS time of low miR-877-5p expression group was significantly shorter than that of high miR-877-5p expression group ($p = 0.0041$) (Figure 2). In addition, patients with low miR-877-5p expression levels displayed lower DFS rates than patients with high miR-877-5p expression levels ($p = 0.0005$). Subsequently, we performed multivariate analysis to further determine the prognostic value of miR-877-5p in HCC patients. We demonstrated that miR-877-5p expression was an independent

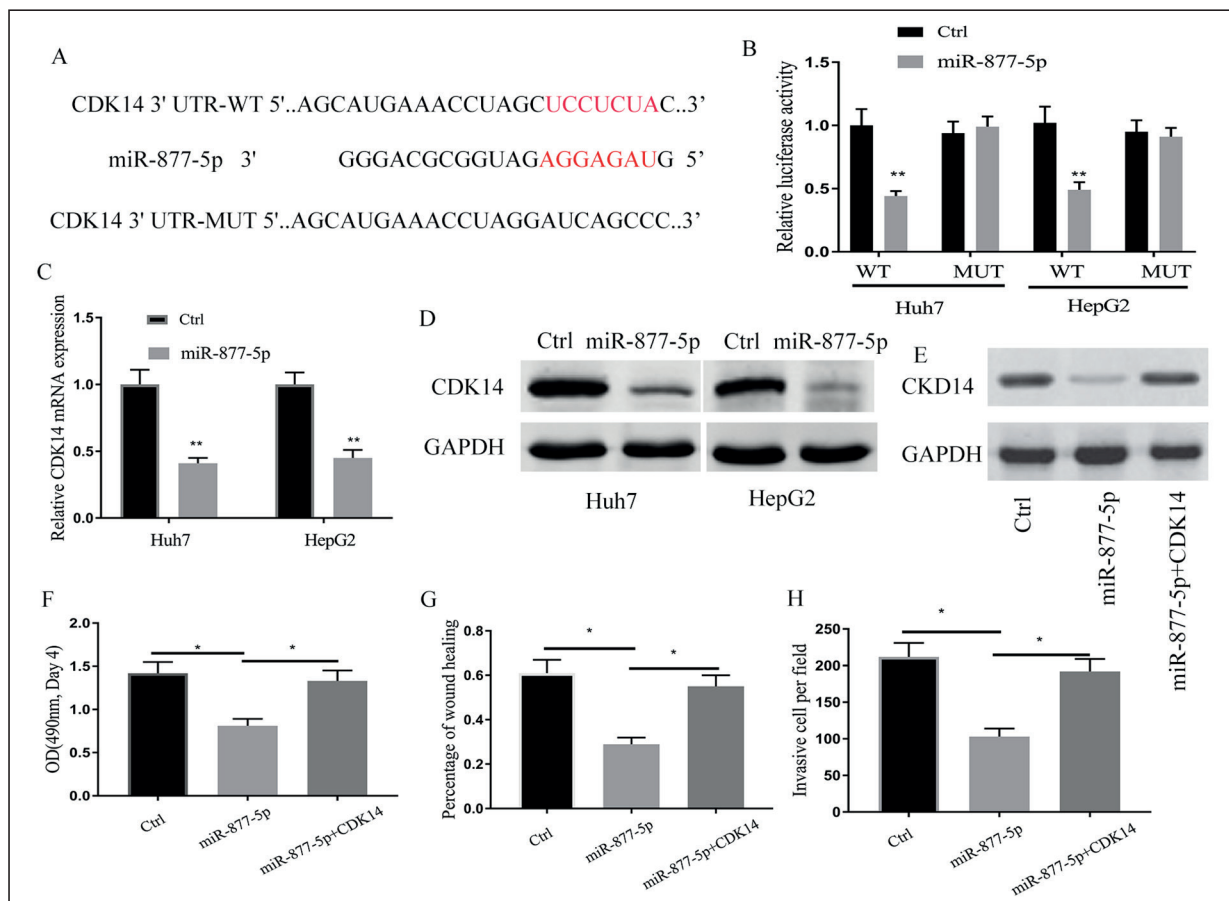


Figure 2. CDK14 was a direct functional target of miR-877-5p in HCC cells and its overexpression influenced the effects of miR-877-5p on HCC cells. (A) Position of the miR-877-5p target site in 3'-UTR of CDK14 mRNA was predicted by microRNA target databases. (B) CDK14-3'-UTR-WT or CDK14-3'-UTR-MUT plasmids were transfected into miR-877-5p-treated Huh7 and HepG2 cells and assayed for luciferase activity. (C) qRT-PCR showing relative level of CDK14 mRNA in transfected Huh7 and HepG2 cells. (D) Relative CDK14 protein level in transfected Huh7 and HepG2 cells based on the Western blot result. (E) CDK14 protein expression in Huh7 cells transfected with negative control, miR-877-5p, or miR-877-5p + CDK14 plasmid. MTT (F), Wound healing (G) and transwell invasion (H) assays were performed in above cells. * $p < 0.05$, ** $p < 0.01$.

prognostic factor for OS time (HR, 3.219; 95% CI, 1.337-7.745; $p = 0.004$) and DFS time (HR, 3.548; 95% CI, 1.561-8.845; $p = 0.001$) in patients with HCC (Table III).

Overexpression of miR-877-5p Inhibited Growth, Migration and Invasion of HCC Cells

In order to explore the influence of miR-877-5p in HCC cells, miR-877-5p mimics or control non-targeting miRNA (Ctrl) was transformed into Huh7 and HepG2 cells. Successful re-expression of miR-877-5p was verified by qRT-PCR (Figure 3A). The results of MTT showed that forced expression of miR-877-5p suppressed the Huh7 and HepG2 cell proliferation (Figure 3B and 3C). In addition, the results of colony formation assay also confirmed that up-regulation of miR-877-5p significantly decreased the clonogenic survival of Huh7 and HepG2 cells compared with their control cells (Figure 3D). To further explore miR-877-5p function on metastasis of HCC, we performed the wound healing and matrigel chamber assays. The results indicated that overexpression of miR-877-5p in Huh7 and HepG2 cells significantly inhibited cell migration (Figure 2E) and invasion (Figure 2F). These data revealed that miR-877-5p was involved in regulating the proliferation, migration and invasion of HCC cells.

MiR-877-5p Directly Targeted CDK14 3'UTR and Inhibited its Expression

In order to further investigate the mechanisms responsible for the effect of miR-877-5p on proliferation and metastasis of HCC cells, we next sought to identify the molecular targets of miR-877-5p. We used a series of algorithms and identified CDK14 as a potential target gene (Figure 2A). Then, we performed a dual-luciferase

reporter assay to confirm whether CDK14 was a potential target gene of miR-877-5p. We found that miR-877-5p remarkably reduced the luciferase activity of wild-type CDK14 3'UTR vector, whereas the luciferase activity of mutant CDK14 3'UTR vector abolished the suppressive effect (Figure 2B). Furthermore, the results of RT-PCR and Western blot indicated that miR-877-5p mimics were able to decrease the expression level of CDK14 at mRNA and protein levels in both Huh7 and HepG2 cells (Figure 2C and 2D).

Overexpression of CDK14 Reverses the Inhibitory Effects of miR-877-5p

In addition, we performed *in vitro* to validate whether miR-877-5p exhibited its tumor-suppressive role by targeting CDK14. CDK14 cells were transfected with negative control, miR-877-5p, or miR-877-5p plus CDK14 plasmid. The results of Western blot showed that overexpression of miR-877-5p suppressed the levels of CDK14, while up-regulation of CDK14 reversed this effect (Figure 2E). Moreover, *in vitro* assay suggested that up-regulation of miR-877-5p inhibited HCC proliferation, migration and invasion, while overexpression of CDK14 reversed the cell inhibitory effect of miR-877-5p (Figure 2F-2H). Our data supported that CDK14 may be a downstream functional mediator of miR-877-5p.

Discussion

MiRNAs play an important role in development and progression in various tumors¹⁷. In addition, more and more evidence showed that miRNA could be used as potential biomarkers for predicting prognosis of tumor patients¹⁸⁻²⁰. However, their potential mechanisms in HCC remained to

Table III. Multivariate Cox proportional hazard model analysis of overall survival and disease-free survival in HCC patients.

	Overall survival			Disease-free survival		
	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>
Gender	0.823	0.543-1.933	0.231	0.934	0.632-2.131	0.197
Age	1.341	0.562-2.342	0.513	1.534	0.673-2.667	0.267
Tumor size	1.732	0.723-2.421	0.423	1.534	0.563-2.017	0.542
Tumor number	1.664	0.789-3.342	0.177	1.983	0.932-3.774	0.194
AFP	1.775	0.723-4.343	0.232	1.932	0.645-5.234	0.138
Hepatitis B	0.899	0.573-2.129	0.217	0.997	0.646-2.451	0.234
Histologic grade	2.943	1.442-6.782	0.006	3.345	1.648-8.841	0.003
TNM stage	2.423	1.538-4.348	0.012	2.783	1.678-5.557	0.008
miR-877-5p expression	3.219	1.337-7.745	0.004	3.548	1.561-8.845	0.001

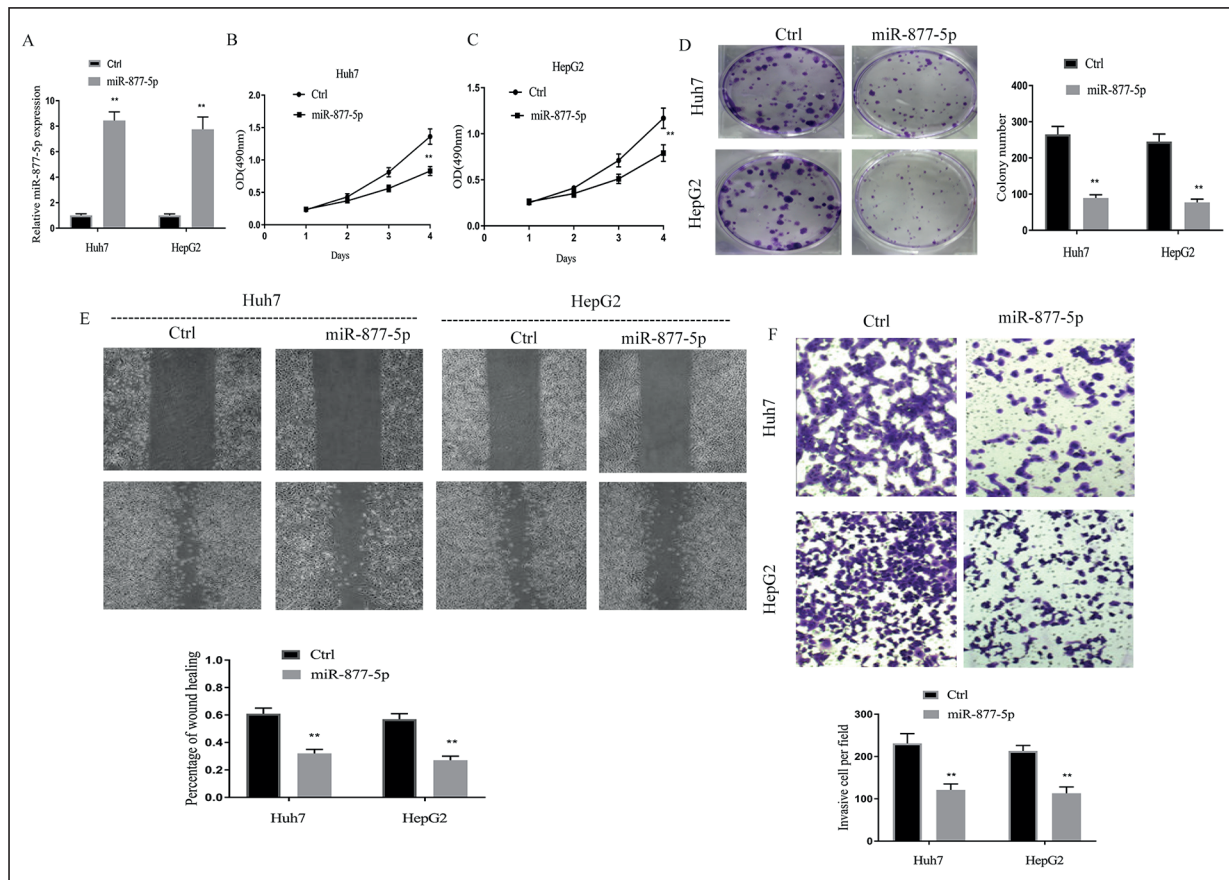


Figure 3. Overexpression of miR-877-5p inhibited cell proliferation, migration and invasion of HCC. (A) The expression of miR-877-5p in Huh7 and HepG2 after treated with miR-877-5p mimic was measured using qRT-PCR. (B, C) The MTT assay was used to measure cell proliferation capacity in Huh7 and HepG2 cells. (D) Colony-forming growth assays were performed to determine the proliferation of Huh7 and HepG2. (E) Wound-healing assay was used to determine cell migratory capability. (F) Transwell assay was performed to determine cell invasive capability. * $p < 0.05$, ** $p < 0.01$.

be elucidated. In the present investigation, we found that miR-877-5p was significantly down-regulated in HCC tissues. Besides, down-regulation of miR-877-5p was also observed in HCC cell lines compared to normal cells. Then, we explored the clinical value of miR-877-5p and found that low miR-877-5p expression correlated with histologic grade and TNM stage, suggesting that miR-877-5p contributed to the progression of HCC. Moreover, survival assay revealed that low miR-877-5p expression was associated with shorter OS and DFS. Besides, multivariate analyses showed that down-regulation of miR-877-5p was an independent factor for predicting OS and DFS in HCC patients. To our best knowledge, this is the first time to report the prognostic value of miR-877-5p in HCC patients.

The function of miR-877-5p in several tumors has been reported in previous studies. For instance, Li et al¹⁵ reported that miR-877-3p served

as a tumor suppressor in bladder cancer because its overexpression could inhibit the bladder cancer growth via activating the expression of p16 gene. Shi et al¹⁴ found that miR-877 suppressed renal cell carcinoma cell proliferation and migration by modulating the eEF2K/eEF2 signaling cascade. More importantly, Huang et al¹⁶ showed that the expression levels of miR-877 was significantly down-regulated in HCC patients. *In vitro* assay indicated that forced miR-877 expression inhibited HCC cell proliferation though targeting FOXM1. At the same time, they found paclitaxel could increase the levels of miR-877. In line with these results, our present study also found that miR-877-5p upregulation could inhibit HCC proliferation, migration and invasion. Thus, combining two results, we confirmed miR-877-5p as a tumor suppressor in HCC progression.

CDK14, also known as PFTK1, is a novel member of Cdc2-related serine/threonine pro-

tein²¹. Growing evidence^{22,23} showed that CDK14 was involved in the regulation of cell cycle progression and cell metastasis by interacting with cyclin D3 and cyclin Y. In addition, dysregulation of CDK14 and its function and mechanism were reported in various tumors. For instance, knock-down of CDK14²⁴ suppressed cell proliferation, metastasis in ovarian cancer by modulating Wnt signaling pathway. High CDK14 expression²⁵ was associated with poor prognosis of gastric cancer and served as a tumor promoter by promoting proliferation, migration and invasion. More importantly, in HCC, CDK14 was reported to promote cells metastasis by inhibiting function of TAGLN2 via phosphorylation and to be a targeting gene of miR-1202^{26,27}. We used three bioinformatics algorithms to predict gene targets for miR-877-5p and found that CDK14 may be a targeting gene of miR-877-5p. The dual luciferase reporter gene assay further confirmed that CDK14 was a target gene of miR-877-5p. Moreover, in vitro assay revealed that up-regulation of CDK14 can rescue the cellular effects induced by miR-877-5p, further confirmed that CDK14 was a target for miR-877-5p. Taken together, our results indicated that miR-877-5p exhibited its anti-tumor function by targeting CDK14.

Conclusions

We demonstrated for the first time that the low level of miR-877-5p correlated with malignant clinical parameters of HCC patients and shortened survival. MiR-877-5p can function as a novel player with metastasis suppressor functions in HCC progression. Our data revealed that the miR-877-5p /CDK14 axis might represent a potential therapeutic strategy for treatment of human HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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