

Original Article

Prognostic significance and anti-proliferation effect of microRNA-365 in hepatocellular carcinoma

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Abstract: MicroRNA functions as tumor suppressor or promoter in hepatocellular carcinoma (HCC). Researchers have found that miR-365 expression was lower in HCC tissues compared with that in adjacent normal tissues. However, its prognostic significance and anti-proliferation effect in HCC remain to be clarified. In this study, we firstly found that miR-365 expression was lower in HCC tissues compared with that in adjacent normal tissues. Then, we analyzed miR-365 expression level and its clinicopathological and prognostic significance. Finally, overexpression of miR-365 inhibits HCC cell proliferation and migration *in vitro*. Our findings suggest that miR-365 expression was an independent poor prognostic factor for HCC patient overall survival and suppressed tumor cell growth. Therefore, miR-365 may serve as a valuable prognostic marker and promising target for HCC.

Keywords: miR-365, hepatocellular carcinoma, prognosis, proliferation

Introduction

Hepatocellular carcinoma (HCC) is the major pathologic type of liver cancer and the third most common cause of cancer death worldwide, with high incidence and death rates [1-6]. According to the statistics, approximately 750,000 new cases are diagnosed and 700,000 people die from it [7]. HCC is difficult to diagnose in early-stage and characterized by high frequency of recurrence, metastasis after surgical resection, and resistance to common chemotherapy and radiotherapy, resulting in poor survival [5, 6]. Despite recent diagnostic and therapeutic advancements, poor prognosis is observed in a large portion of HCC patients. However, overall patient survival remains low [7]. Especially, the five year survival rate for late stage liver cancer is still well below 10% [8].

MicroRNAs (miRNAs) belong to non-coding small RNAs of approximately 22 nucleotides and regulate the translational inhibition of target mRNAs by base-pairing with their 3'-untranslated region (3'-UTR) [9-11]. The abnormal expression of miRNAs has been demonstrated in many types of cancer, whereby

miRNAs function as oncogenes or tumor suppressors during tumor development and progression, indicating their potential as biomarkers for diagnosis and treatment [12-15]. Over the past few decades, growing evidence suggests that dysregulation of miRNAs may contribute to various types of cancers, including HCC [16]. In a previous study, researchers found that the expression of miR-365 was significantly downregulated compared with that in matched normal tissue [17]. However, the correlation between the expression of miR-365 and clinicopathological parameters in HCC patients, and the exact mechanisms of miR-365 in HCC development have not yet been previously reported.

In this study, we firstly demonstrated that miR-365 expression was lower in HCC tissues compared with that in adjacent normal tissues. Next, we analyzed miR-365 expression level and its clinicopathological and prognostic significance. Finally, we found that overexpression of miR-365 remarkably suppressed proliferation and migration capacities of HCC cell *in vitro* experiments. Our findings will help to elucidate the functions of miRNAs and their roles in tumorigenesis.

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Materials and methods

Patients

All patients had signed informed consent forms and the study was approved by the Ethics Committee of The Third Xiangya Hospital of Central South University. The selection criteria for patients with HCC were as follows: (1) Pathologically confirmed patients with HCC and two pathologists respectively reviewed all of the cases. (2) The patients haven't received preoperative anti-tumor therapy. (3) The patients had no other cancer history. All patients were diagnosed and treated between May 2006 and March 2014. The mean follow-up period was 41 months. Clinical staging was performed according to the 6th edition 2002 American Joint Committee on Cancer (AJCC) TNM staging system. Survival time was calculated from the date of the initial surgery to death.

Tissue specimens and cell lines

Human HCC and corresponding normal tissues (located > 2 cm away from the tumor) were obtained from 15 patients who underwent HCC resection at Xiangya Hospital Central South University. The other 32 cases of HCC tissue for test of miRNA-365 expression level were collected from the pathology department of The Third Xiangya Hospital of Central South University. Human HCC cell line HepG2 was purchased from the ATCC (Manassas, VA, USA). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂ in a humidified atmosphere.

Quantitative real-time RT-PCR

miRNA from tissues and cells was extracted using TRIzol (Invitrogen) reagent according to the manufacture's instruction. Real-time qRT-PCR was carried out as described previously [18]. The qRT-PCR primers for miR-365 were forward 5'-CGTAATGCCCTAAAAAT-3' and reverse 5'-GTGCAGGGTCCGAGGT-3'. Internal control U6 primers were forward 5'-CTC-GCTTCGGCAGCAC-3' and reverse 5'-AACGC-TTCACGAATTTGCGT-3'. The relative expression level of miR-365 was normalized to that

of U6 by using the 2^{-ΔΔCt} cycle threshold method [11].

Cell transfection

Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Both miR-365 mimics and miRNA NC mimics were purchased from GenePharma (Shanghai, China). HepG2 cells were seeded and transfected with 50 nM miR-365 mimics or miRNA NC mimics. 48 h after transfection, the fluorescence microscopy and the qRT-PCR were used to check transfection efficiency.

Cell proliferation assay

The cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) colorimetric assay was performed to detect cell proliferation. 48 h after transfection, cells were seeded in 96-well plates by 5 × 10³ cells per well. Three repeated wells were set in each group. CCK-8 (10 μl) was added to each well at 24, 48, 72 h later, and incubation was performed at 37°C for 2 h. An ELIASA was used to detect absorbency value at 450 nm.

For the colony formation assay, HepG2 cells were seeded in 10 cm dishes (1000/plate) after transfection and maintained in complete culture medium for 21 days. Next, cells were fixed in 4% paraformaldehyde for 15 min and stained with GIMSA dye. Cells were photographed and the number of clones was calculated.

Scratch test

Scratch test was performed to confirm the influence of miR-365 on HCC cell migration. Cells were collected and seeded in 6-well plates at 5 × 10⁵/well and cultured until a complete monolayer was achieved. And then the plate was scratched a straight line using a 1 mL tip. The migration of cells into the scratch-generated gap was observed under microscope cultures at 0, 24 and 48 h.

Statistical analysis

Statistical analyses were carried out using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad

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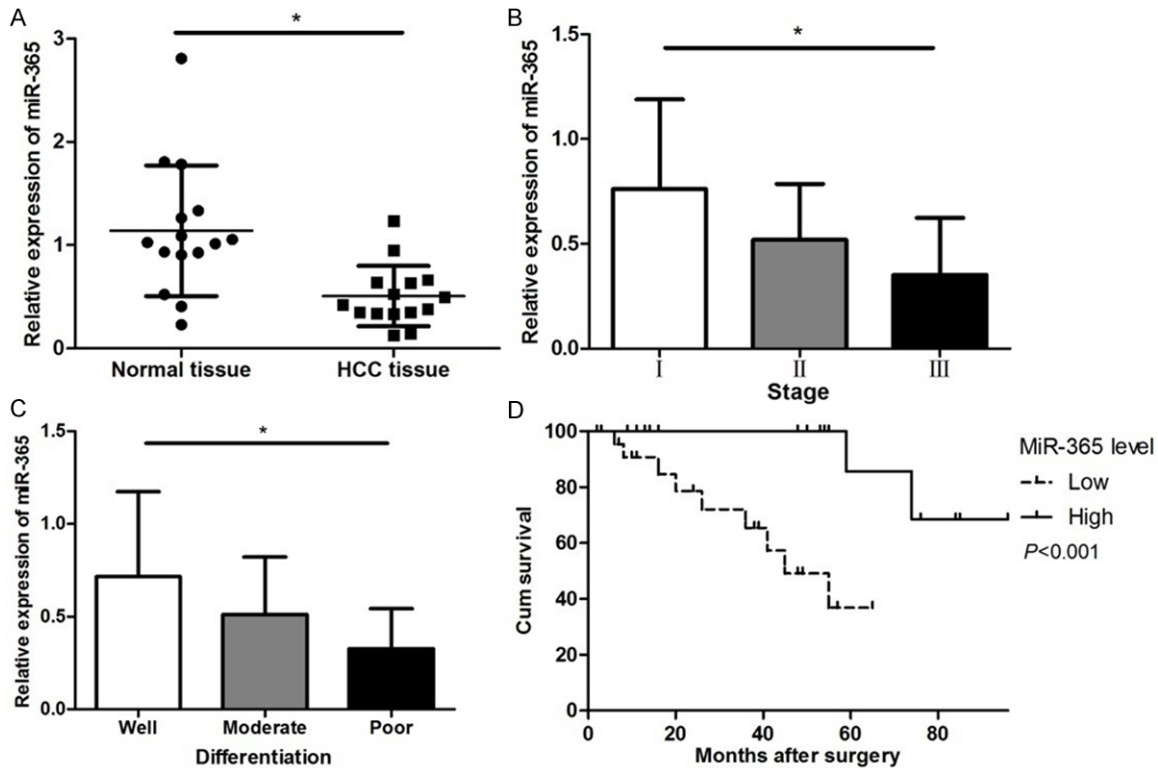


Figure 1. Expression levels of miR-365 and clinicopathological characteristics in HCC. A. Comparison of miR-365 expression levels between HCC tissues and adjacent normal tissues. B. The expression level of miR-365 in HCC patients at different clinical stages. C. The expression level of miR-365 at different differentiation of HCC tissues. D. Overall survival curve of HCC patients with different miR-365 expression. The patients with a lower expression of miR-365 had a lower survival time compared with those with a higher expression of miR-365. (* $P < 0.05$).

Software Inc., CA, USA). Difference of measurement data was assessed by Student's t-test or one-way ANOVA. Count data were analyzed by the χ^2 or Fishers exact tests. Univariate survival analysis was performed using Kaplan-Meier method and the Log-rank test. Multivariate survival analysis was performed using the Cox multivariate analysis model. $P < 0.05$ was considered statistically significant.

Results

Expression levels of miR-365 in HCC

We used qRT-PCR to examined miR-365 expression in 15 pairs of HCC tissues and the corresponding noncancerous tissues. As shown in **Figure 1A**, the expression of miR-365 was significantly down-regulated in HCC tissues when compared with para-cancer tissues. The miR-365 level was significantly negatively correlated with clinical stage in HCC tissue specimens (shown in **Figure 1B**). MiR-365 expression in HCC tissues with various grades of differentia-

tion was also remarkably different (shown in **Figure 1C**).

Expression levels of miR-365 and clinicopathological characteristics in HCC

The miR-365 expression levels were classified as high or low in relation to the median value. Low expression of miR-365 was found to significantly correlate with tumor size, clinical stage and tumor differentiation. However, there were no significant difference in miR-365 expression was observed with age, sex distribution, tumor number, cirrhosis and serum AFP value shown in **Table 1**).

Low-expression level of miR-365 predicts poor prognosis in HCC patients

Considering that the level of miR-365 expression was remarkably correlated with tumor size, clinical stage and tumor differentiation, we hypothesized that miR-365 might affect the prognosis of HCC patients. Kaplan-Meier analy-

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Table 1. Correlation between the expression of miR-365 and clinicopathological parameters in HCC patients

Characteristics	Cases	n%	miR-365 expression		P value
			High (n = 24)	Low (n = 23)	
Age (years)	< 50	24	65.22	11	0.464
	≥ 50	23	34.78	13	
Sex distribution	Male	30	65.22	13	0.159
	Female	17	34.78	11	
Tumor number	Solitary	39	82.98	19	0.477
	Multiple	8	17.02	5	
Tumor size (cm)	≤ 5	24	39.13	16	0.029
	> 5	23	45.65	8	
Vascular invasion	Yes	11	23.40	3	0.071
	No	36	76.60	21	
Clinical stage	I	11	23.91	8	0.018
	II	17	36.96	11	
	III	19	39.13	5	
Differentiation	Well	14	30.43	11	0.002
	Moderate	19	39.14	11	
	Poor	14	30.43	2	
Cirrhosis	Negative	27	60.67	14	0.900
	Positive	20	39.13	10	
AFP (μg/L)	< 30	16	32.61	6	0.181
	≥ 30	31	67.39	18	

Table 2. Multivariate analyses for overall survival by Cox regression model

	Wald	P-value	RR	RR 95% CI	
				Lower	Upper
Age	0.559	0.455	0.398	0.036	4.45
Gender	0.785	0.384	2.192	0.374	12.831
Tumor size	2.011	0.156	0.136	0.009	2.144
Vascular invasion	1.566	0.211	0.224	0.022	2.332
Clinical stage	5.842	0.016	56.803	2.146	1503.286
Differentiation	3.256	0.071	14.643	0.793	270.258
Cirrhosis	5.62	0.018	283.419	2.659	30203.530
AFP	2.529	0.112	0.039	0.001	2.126
miR-365	4.523	0.033	0.003	0.000	0.637

sis with the log-rank test indicated that low miR-365 expression had a significant impact on OS (36.84% vs. 68.57%; $P < 0.001$; **Figure 1D**). Univariate and multivariate analyses were utilized to evaluate whether the miR-365 expression level and various clinicopathological features were independent prognostic parameters. Multivariate analysis revealed that miR-365 expression, clinical stage, tumor differentiation and cirrhosis were independently associated with the overall survival (shown in **Table 2**).

Overexpression of miR-365 inhibits HepG2 cell proliferation ability

In order to further investigate the role of miR-365 in HCC, miR-365 mimics or NC mimics (**Figure 2**) was transfected into HepG2 cells. To elucidate whether miR-365 suppressed HepG2 cell proliferation, CCK-8 assays were employed. At 24, 48, 72 and 96 h, the proliferation rates in miR-365 mimic-transfected group (miR-365-HepG2) was significantly slower compared with either the untreated group (HepG2) or control (NC) (**Figure 3A**). This suggested that the up-regulation of miR-365 expression inhibits HepG2 cell proliferation ability. Colony formation assays were also used to evaluate cell proliferation and plating efficiency after overexpression of miR-365. The results showed that the number of colonies formed of miR-365 group was much lower than that in HepG2 and NC groups (**Figure 3B**). The results of the CCK-8 and colony formation assays therefore demonstrate that miR-365

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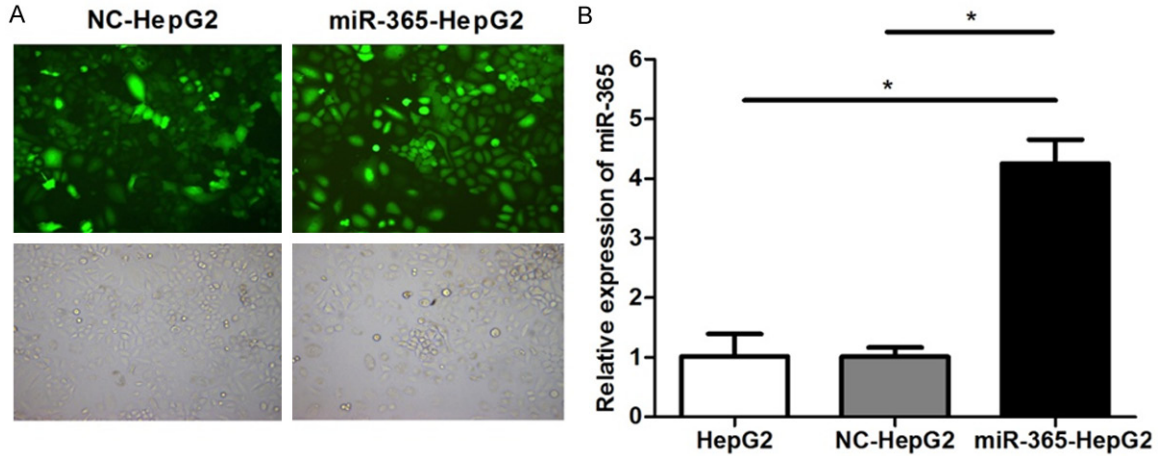


Figure 2. Overexpression of miR-365 in HepG2 cell. HepG2 cells were untransfected (HepG2) or transfected with NC mimics (NC-HepG2) or miR-365 mimics (miR-365-HepG2). A. Representative fluorescence microscopy images ($\times 100$, top) and bright field microscopy images ($\times 100$, bottom) are at left. B. Validation of miR-365 overexpression in HepG2 cells by qRT-PCR analysis. ($*P < 0.05$).

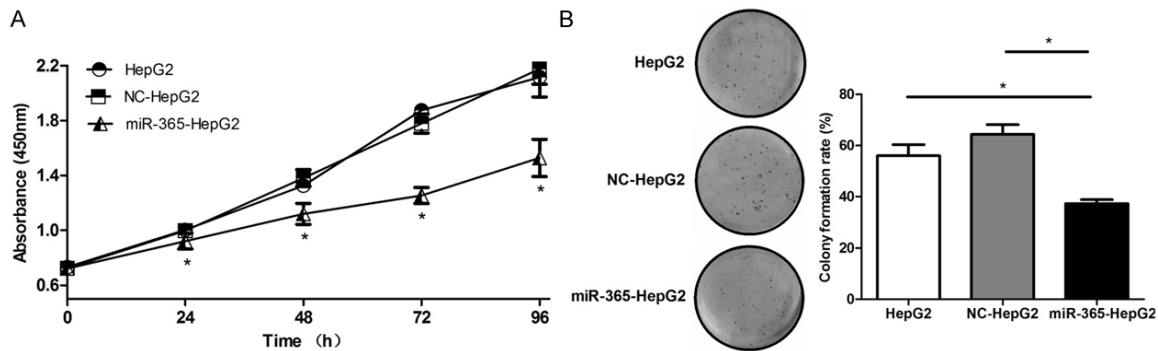


Figure 3. Overexpression of miR-365 inhibits HepG2 cell proliferation. A. CCK-8 assays were performed to examine HepG2 proliferation at the indicated time points. B. Colony formation assays were performed to evaluate HepG2 cell proliferation. ($*P < 0.05$).

overexpression limits HepG2 proliferation suggesting that it may inhibit HCC growth *in vivo*.

Overexpression of miR-365 inhibits HepG2 cell migration ability

To evaluate the effect of miR-365 interaction on migration of HCC cells, scratch test was used. The results of the scratch test showed that the closure of the gap area of miR-365 group was significantly less than that of HepG2 and NC-HepG2 groups (Figure 4), which indicated that the migratory ability of HepG2 cells was inhibited by miR-365.

Discussion

A large number of studies suggest that understanding of miRNA function will provide us

broad prospects to understand process of tumorigenesis and the behavior of cancer cells. Increasing evidence suggests that the dysregulation of miRNAs participates in HCC progression. For example, Zhang Y [19] *et al.* demonstrated that the down-regulation of miR-101 in clinical HCC tissues associates with tumor aggressiveness and worse survival of HCC patients. MiR-29b [20] and MiR-122 [21] have been characterized to have anti-metastatic and anti-angiogenic functions in HCC.

Reduced expression of miR-365 has been observed in different types of cancers. MiR-365 has been shown to downregulate expression in lung cancer and inhibit lung cancer cell proliferation through regulating NKX2-1 [22]. MiR-365 induces gemcitabine resistance in pancreatic cancer cells by targeting the pro-

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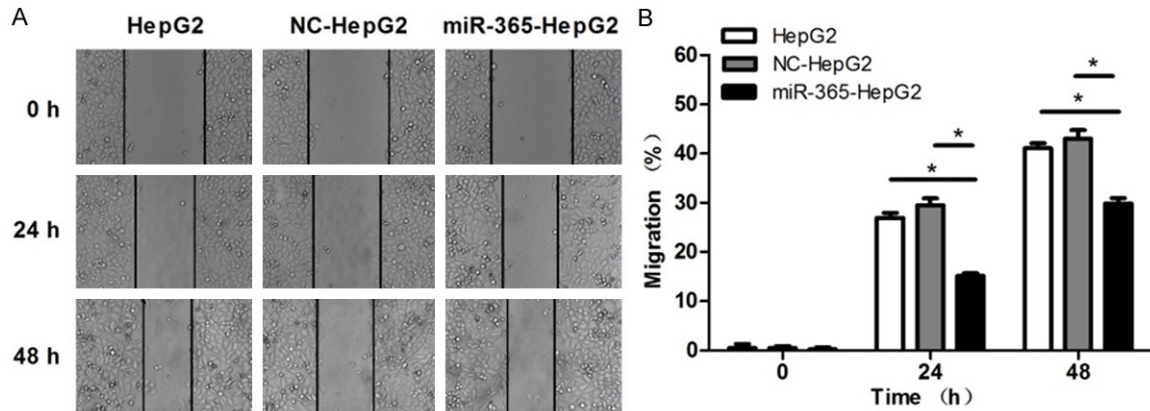


Figure 4. Overexpression of miR-365 inhibits HepG2 cell migration. Representative microscopic images of scratch-induced gap at the indicated time points ($\times 40$) are on the left. Migration rate of each group at 0, 24 and 48 h is showed on the right. (* $P < 0.05$).

apoptotic molecules SHC1 and BAX [23]. However, miR-365 also has been found to promote cutaneous squamous cell carcinoma (CSCC) through targeting NFIB [24]. All these findings emphasize a fundamental role of miR-365 in development of malignant tumor.

Previously, researchers found that 29 microRNAs exhibited significantly differential expression in the HCC tissues compared to that of normal tissues via noncoding RNA microarray analysis, including miR-365 [17]. However, relevant function and mechanism remain to further explore.

In the present study, our results showed that miR-365 expression was significantly lower in HCC tissues compared with normal adjacent tissues. The aforementioned results were consistent with the study results of Hang Su [17] *et al.* The relationship of the miR-365 with various clinical features of HCC was analyzed. The results revealed that a low level of miR-365 expression was significantly correlated with tumor size, clinical stage and tumor differentiation, suggesting that miR-365 might be closely related with carcinogenesis and tumor development of HCC. Furthermore, the survival rate of low miR-365 expression group was significantly shorter than that of high miR-365 expression group. In a multivariate Cox model, we found that miR-365 expression was an independent poor prognostic factor for overall survival rate, indicating that low miR-365 level was significantly associated with poor OS in HCC patients. In addition, in order to know more about the effect of miR-365 *in vitro*, we overexpressed

miR-365 in HepG2 cells. The cell proliferation and colony formation assays revealed that miR-365 suppresses proliferation of HepG2 cells. The results of scratch test showed that miR-365 inhibits cell migration ability. In future studies, we will collect more HCC tissues of patients and explore the targeted genes of miR-365 and downstream signaling pathways.

In conclusion, the expression of miR-365 was decreased in HCC. Low expression of miR-365 was significantly associated with tumor progression and decreased survival in patients with HCC. What's more, miR-365 inhibited HCC cell proliferation and migration. These findings indicate that miR-365 serves as a novel prognostic marker and tumor suppressor in HCC.

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Disclosure of conflict of interest

None.

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