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miR-630 Overexpression in Hepatocellular Carcinoma Tissues is Positively Correlated with alpha-Fetoprotein

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Background: MicroRNA-630 (miR-630) has been shown to be involved in various human malignancies. However, its role in hepatocellular carcinoma (HCC) remains unknown.

Material/Methods: TaqMan qRT-PCR assay was performed to detect the expression of miR-630 in 42 pairs of HCC tissues and corresponding noncancerous hepatocellular tissues, and its correlations with clinicopathologic features and serum alpha-fetoprotein (AFP) level of patients were analyzed.

Results: The present study found that miR-630 expression was significantly increased in HCC tissues and cells compared with their normal counterparts. miR-630 expression level did not significantly change at stage I but was markedly increased at advanced TNM stage (stage II-III). In addition, the increased expression of miR-630 in tissues of HCC appeared in patients who exhibited elevated serum levels of AFP (>25 ng/ml), but not in those with normal AFP levels (≤25 ng/ml). The miR-630 expression in carcinoma tissues revealed a positive correlation with the levels of serum alpha-fetoprotein (AFP; $R^2=0.768$).

Conclusions: These results suggest that miR-630 is associated with tumor progression of hepatocellular carcinoma and may be a potential prognosis indicator.

MeSH Keywords: **alpha-Fetoproteins • Carcinoma, Hepatocellular • MicroRNAs**

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Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with very high morbidity and mortality [1]. The prognosis of HCC is poor and most patients are generally diagnosed at advanced stage [2,3]. Approximately 600,000 HCC patients die annually, making it the fifth most common cause of cancer-related death worldwide [4]. The 5-year survival rate is about ~50%, and that of the patients with frequent intra- or extra-hepatic metastasis is less than 5% despite recent advances in surgical techniques and medical treatment [5–7]. Although previous studies identified many aberrantly expressed genes in HCC, novel molecular markers are still urgently needed to help in early diagnosis and risk assessment [8,9]. Moreover, investigating the relationships between molecular changes and clinical symptoms is of paramount importance for developing new diagnosis and treatment strategies for HCC and improving the prognosis of patients.

MicroRNAs (miRNAs), small non-coding endogenous RNA gene products consisting of 18 to 25 nucleotides, were identified as important regulators of malignant transformation [10–12]. Recently, by using detection analyses such as microarray and RNA sequencing, various miRNAs were identified as aberrantly expressed in human HCC [13–15], some miRNAs act as oncogenes or tumor suppressors, and play critical roles in many aspects of HCC carcinogenesis and progression, including cell proliferation, apoptosis, angiogenesis, and metastasis [16,17]. In addition, based on this deregulated miRNAs expression profiling and their association with the biological and clinical properties of HCC, specific miRNAs could be utilized to distinguish benign from malignant lesions [18–21], and others may be powerful predictors of clinical outcomes and therapeutic molecular targets in HCC [22,23].

MiR-630, identified from the microRNA cluster site at chromosome 15q24.1, has been reported to be deregulated and to be involved in tumor progression of several human malignancies [24–29]. For example, miR-630 has been reported to regulate cisplatin-induced lung cancer cell death [24]; in head and neck squamous cell carcinoma cells exposed to cisplatin, miR-630 is involved in the apoptotic process and autophagic pathways by modulate protein expression of multiple genes including ATG5, ATG6/BECN1, ATG10, ATG12, ATG16L1, and UVRAG [25,26]. Moreover, by targeting IGF-1R, miR-630 could induce apoptosis of pancreatic cancer cells, reduced breast cancer cellular aggression, and improve patient response to HER-targeting drugs [27,29]. miR-630 was shown to be closely associated with poor overall patient survival in clear cell renal cell carcinoma [30], colon cancer [31], and gastric cancer [32]. All these results indicate that by its involvement in different signaling pathways, miR-630 is able to modulate multiple pivotal processes of various human cancers. However, little is known about the expression level and clinical significance of miR-630 in HCC.

In the current study, we clarified the clinical significance of miR-630 expression in HCC. The primary aim of this study was to investigate whether miR-630 is detectable and altered in HCC tissues or cell lines compared with adjacent normal tissues or normal cell lines. Then, we investigated a potential relationship between this miRNA level in tumor tissues and existing clinicopathological features of HCC, such as tumor size, number, histologic stage, serum levels of AFP, HBeAg, HBsAg, ALT, the status of vascular invasion, cirrhosis, and encapsulation.

Material and Methods

Clinical samples collection

In total, we selected 42 patients (aged 39–75 years; mean, 56.0 years) diagnosed with primary HBV-related HCC who underwent hepatectomy, without preoperative chemoradiotherapy, at the Third Affiliated Hospital of Sun Yat-sen University between December 2011 and April 2013. All resected HCC tissues and corresponding noncancerous hepatic tissue specimens were immediately frozen in liquid nitrogen and stored in -80°C . Paired noncancerous tissues were isolated from at least 2 cm away from the tumor border and were shown under microscopy to be without tumor cells. All tumor nodules were verified by pathological examination. Clinical indexes such as serum levels of AFP, HBeAg, HBsAg, ALT, and the number, size, and grade of tumor foci were determined, and complete clinical-pathologic and follow-up data were available. Tumor staging was defined according to the sixth edition of the tumor node metastasis (TNM) classification system published by the International Union Against Cancer and the Barcelona Clinic Liver Cancer (BCLC) staging system. The study was approved by our Institutional Review Board, and informed consent was obtained from all patients and controls.

Cell cultures

HCC cell lines SMMC-7721, Hep3B, HepG2, HCCLM3, and LO2 were cultured at 37°C in an atmosphere containing 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (v/v), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 1% non-essential amino acids (v/v) (Invitrogen Life Technologies, Carlsbad, CA, USA) and cultured at 37°C in an atmosphere of 5% CO_2 with a relative humidity of 95%. Normal hepatic cell line, HL-7702, was grown in RPMI-1640 medium, supplemented with heat-inactivated 10% FBS.

RNA extraction and quantitative reverse transcription (RT)-qPCR

Total RNA was extracted from frozen cancer tissues using TRIzol[®] reagent (Invitrogen Life Technologies, Carlsbad, CA,

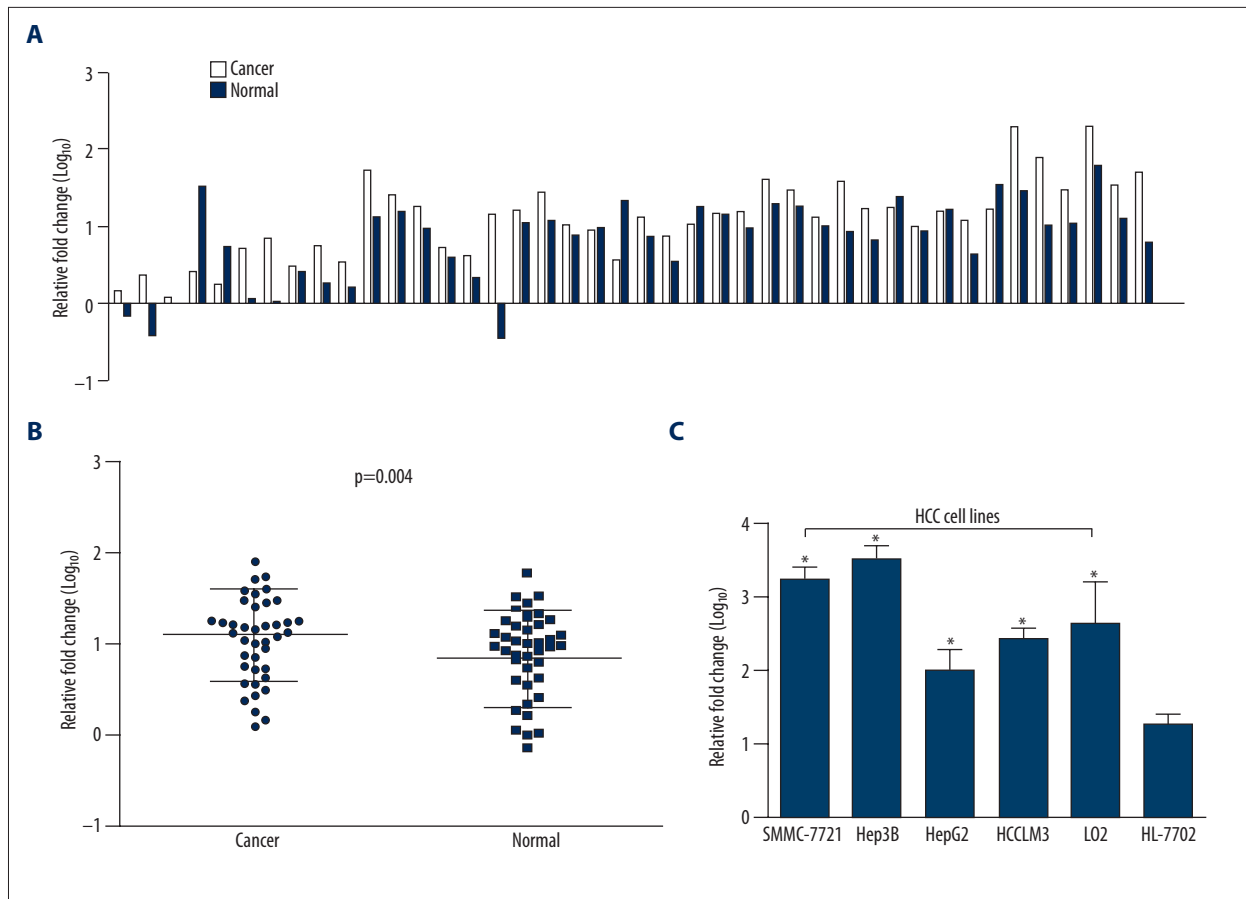


Figure 1. Expression of miR-630 in hepatocellular tissues and cultured hepatocellular cells. miR-630 expression was detected in (A, B) hepatocellular carcinoma tissues (n=42) and (C) various hepatocellular carcinoma cells (n=5) by RT-qPCR. Adjacent noncancerous tissues (adjacent) and normal hepatocellular HL-7702 cells were used as controls. * P<0.05, vs. the HL-7702 group.

USA) according to the manufacturer's instructions. cDNA was reverse transcribed from the total RNA samples using specific miRNA primers from the TaqMan MicroRNA Assays and reagents from the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). The cDNA was amplified by PCR using TaqMan MicroRNA Assay primers with the TaqMan Universal PCR Master Mix and analyzed with an Eppendorf Mastercycler EP Realplex (Eppendorf, Germany). The relative levels of miRNA expression were calculated from the relevant signals by normalization with the signal of U6 snRNA expression. miRNA expression levels were calculated based on $LOG_{10}2^{-\Delta\Delta Ct}$, and were analyzed as mean \pm SD.

Serum AFP determination

Serum AFP levels were measured using ELISA (no. ab3980; Abcam, Cambridge, UK), and dilutions of excessively high AFP concentration, setup, adjustments and quality controls were performed according to the manufacturer's instructions. The assay employed anti-human AFP antibody coated

onto a 96-well plate. Standard or serum samples were pipetted into the wells and AFP present in samples was bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-human AFP antibody was added. Following washing of unbound biotinylated antibody, horseradish peroxidase-conjugated streptavidin was pipetted into the wells. The wells were washed again, TMB substrate solution was added, and color developed in proportion to the amount of bound AFP. Absorbance value was measured at 450 nm. AFP concentration in serum was achieved according to standard curves.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL, USA). The significance of differences between groups was analyzed using the *t* test, χ^2 test, or Fisher's exact test, as appropriate. Correlations between miR-630 and serum AFP levels were calculated by linear regression analysis. P<0.05 was considered statistically significant.

Results

Increased levels of miR-630 in HCC tissues and cells

In order to explore the level of miR-630 in hepatic carcinogenesis and progression, we detected the expression of miR-630 in HCC tissues and adjacent noncancerous tissues using TaqMan RT-qPCR assay. Our results revealed that the mean expression level of miR-630 in HCC tissues (mean \pm SD: 1.10 ± 0.51) was remarkably higher than that in noncancerous tissues (mean \pm SD: 0.90 ± 0.55 ; $P=0.004$; Figure 1A, 1B). RT-qPCR assays were further developed to quantify miR-630 levels in HCC cell lines, including SMMC-7721, Hep3B, HepG2, HCCLM3, and LO2 cells, and the normal hepatic cell line HL-7702. A significantly higher expression of miR-630 was found in HCC cell lines compared to HL-7702, and HCCLM3 cells exhibited the highest expression of miR-630 (all $p<0.05$, Figure 1B).

Correlation between miR-630 expression and clinicopathologic characteristics of HCC patients

Based on the median (1.14) miR-630 level, all 42 HCC patients were divided into 2 subgroups: a low miR-630 group (≤ 1.14) and a high miR-630 group (>1.14). Our results revealed that miR-630 was significantly associated with TNM stage ($p=0.001$), serum AFP level ($p=0.002$), micro-vascular invasion ($p=0.004$), and macro-vascular invasion ($p=0.031$), but not with other clinicopathologic parameters including age, sex, serum HBeAg, HBsAg and ALT level, tumor differentiation, tumor size, capsulation status, and cirrhosis (all $p>0.05$, Table 1). The expression level of miR-630 in patients was then further analyzed according to TNM stage. Our results show that in patients at stage I there were no significant differences in miR-630 level between carcinoma tissues and adjacent non-tumor tissues ($p>0.05$, Figure 2). However, in patients at stage II and III, compared with adjacent noncancerous tissues, the expression of miR-630 was markedly increased in carcinoma tissues ($p=0.009$ and 0.017 , respectively; Figure 2).

miR-630 positively correlates with the serum AFP level

We further determined the relationship between miR-630 expression and serum AFP level after re-measuring serum AFP levels of all patients using ELISA. The result showed that, consistent with clinical reviewed data (patient serum AFP level was determined using radioimmunoassay method), among all 42 patients, 11 patients exhibited normal serum AFP levels (≤ 25 ng/ml), while the other 31 patients exhibited abnormal serum AFP levels (>25 ng/ml). In adjacent noncancerous tissues, there was no difference of the expression of miR-630 between patients with normal and abnormal serum AFP levels (Figure 3A). However, in carcinoma tissues, while in patients with normal AFP levels the miR-630 expression was not significantly different

from the adjacent noncancerous tissues ($p>0.05$; Figure 3A); a significant increase of miR-630 appeared in those with abnormal AFP levels (1.76-fold vs. the adjacent noncancerous tissues; $p=0.003$; Figure 3A). Correlation analysis result showed that miR-630 levels in carcinoma tissues were positively correlated with serum AFP value ($R^2=0.768$, $p<0.001$; Figure 3B).

Discussion

miRNAs are a family of non-coding small RNA molecules, which reveal complex functional effects by function as protein-coding RNA silencing factors in almost all physiological processes, including differentiation, proliferation and apoptosis [33]. In addition, miRNAs are important regulators in the carcinogenesis and development of various neoplasms including HCC [10–12,16]. miRNA-630 has been previously reported to be up-regulated in various types of cancer tissues and may be an important regulator in tumor progression [24–32,34]. In the present study, we sought to determine whether there was any difference in miR-630 expression between HCC and normal tissue samples. In addition, we investigated the expression level of miR-630 in HCC lines and a normal hepatic cell line. Our results show that HCC tissues and cell lines (SMMC-7721, Hep3B, HepG2, HCCLM3, and LO2 cells) exhibited higher miR-630 levels than their normal counterparts. We believe the present study is the first to report the elevated expression of miR-630 in human HCC tissues and cell lines.

It was found that miR-630 could predict recurrence after surgical resection in stage I non-small cell lung cancer [34], and regulate cisplatin-induced lung cancer cell death [24]. miR-630 expression was upregulated in head and neck squamous cell carcinoma after cisplatin treatment and can modulate the protein expression of ATG5, ATG6/BECN1, ATG10, ATG12, ATG16L1, and UVRAG [25,26]. Recent studies also reported that by targeting IGF-1R, miR-630 could induce apoptosis of pancreatic cancer cells, reduce breast cancer cellular aggression, and enhance and improve patient response to HER-targeting drugs [27,29]. All these findings indicate that miR-630 is an important modulator and may be useful in monitoring the response to treatment. Moreover, miR-630 was also reported as a valuable prognosis predictor for clear cell renal cell carcinoma [30], colorectal cancer [31], and gastric cancer [32]. In the present study, we found the expression level of miR-630 was significantly associated with important supplementary parameters in HCC, including TNM stage, serum α -fetoprotein (AFP), and vascular invasion. To further address the possible role of miR-630 in HCC, we investigated miR-630 expression level according to TNM stage and serum AFP.

Previous studies in gastric and colorectal cancer showed that the high expression of miR-630 was more frequently detected

Table 1. Correlation between miR-630 expression and clinicopathological characteristics in 42 HCC patients.

Variable	miR-630 expression		P value
	Low (n=21)	High (n=21)	
Age (years)			0.500
≤55	9	10	
>55	12	11	
Gender			0.367
Male	16	14	
Female	5	7	
Tumor grade			0.139
I-II	18	14	
III-IV	3	7	
TNM stage			0.001*
I	12	2	
II-III	9	19	
Tumor size (cm)			0.119
≤5	19	15	
>5	2	6	
Tumor number			0.227
Solitary	15	18	
Multiple	6	3	
AFP (ug/L)			0.002*
≤20	10	1	
>20	11	20	
Encapsulation			0.090
No	17	12	
Complete	4	9	
Micro-vascular invasion			0.004*
Absent	12	3	
Present	9	18	
Macro-vascular invasion			0.031*
Absent	13	6	
Present	8	15	
Liver cirrhosis			0.806
Without	10	11	
With	40	39	
HBs antigen			0.376
Negative	7	9	
Positive	14	12	
HBe antigen			0.177
Negative	13	9	
Positive	8	12	
ALT (U/L)			0.242
≤40	4	7	
>40	17	14	

The median expression level of miR-630 was used as the cutoff. Patients with HCC were divided into miR-630 “Low” group (whose expression was lower than the mean) and “High” group (whose expression was higher than the median). * P <0.05.

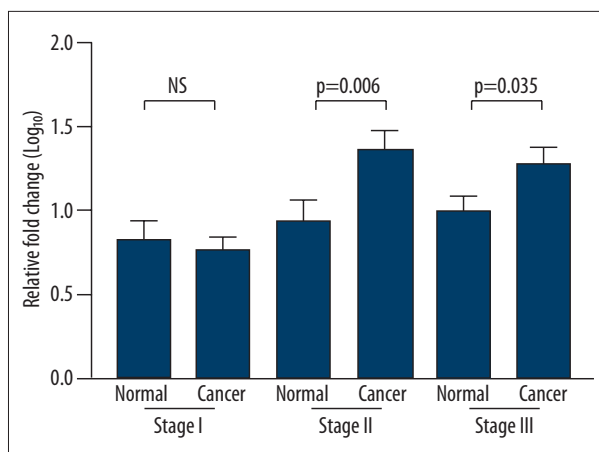


Figure 2. Expression of miR-630 at various TNM stages in hepatocellular carcinoma tissues. miR-630 expression in hepatocellular carcinoma and adjacent noncancerous tissues at TNM stage I (n=14), stage I (n=12) and stage III (n=16) are shown.

in tumors with advanced TNM stage [31,32]. In the present study, the miR-630 expression detected at TNM stage I was not significantly different from the adjacent noncancerous tissues, whereas miR-630 was markedly increased in cancer tissues at stages II and III. According to the International Union Against Cancer and the Barcelona Clinic Liver Cancer (BCLC) staging system, patients at TNM stage II-III all had vascular invasion, which helps to understand how the high expression of miR-630 may be associate with tumor invasion status in our cohort. Our findings suggest that miR-630 may play important roles in HCC carcinogenesis and progression and were consistent with other reports that miR-630 participates in tumor invasion and metastasis [28,29]. The overexpression of

miR-630 may be useful in predicting which patients will have early invasion and metastasis of HCC.

In the current study it was notable that the increase of miR-630 in carcinoma tissues only existed in patients with elevated serum AFP levels (>25 ng/ml). Moreover, miR-630 levels in carcinoma tissues were positively associated with serum AFP levels. Serum AFP is the most widely used biomarker of HCC, and it has been commonly utilized as a categorical variable for staging and as a tool for predicting recurrence and survival [35,36]. Serum AFP is significantly correlated with recurrent tumor size [37]; however, in size-matched cases, patients with low serum AFP levels have been shown to have significantly improved prognosis compared with patients with elevated serum AFP levels [38]. Thus, based on our findings in the present study, increased miR-630 mRNA in carcinoma tissues might be useful in predicting prognosis.

Conclusions

We found that miR-630 expression is increased in hepatocellular carcinoma tissues and is associated with tumor progression. Our results suggest that miR-630 plays an important role in invasiveness and metastasis. Its characteristic expression according to TNM stage and positive correlation with serum AFP levels suggest that it may be a good risk assessment marker of vascular invasiveness and a prognosis indicator in clinical practice. However, since individual miRNA targets different genes in different cancer types, the role of miR-630 in hepatocellular carcinoma (serving as an oncogene/suppressor or not) and possible targets, such as SLUG [28] and IGF-1R [27,29], require further investigation.

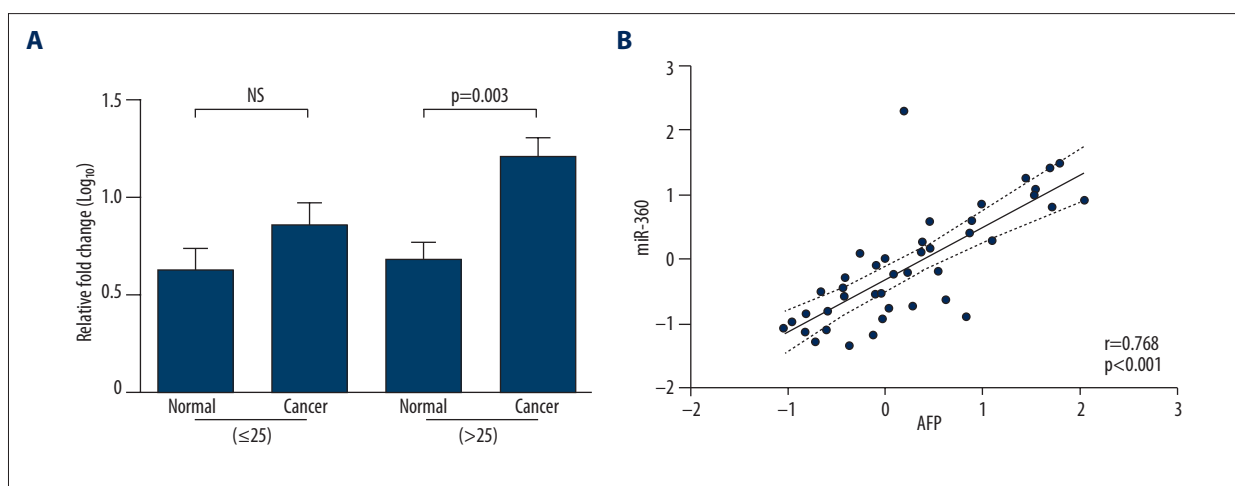


Figure 3. Correlation between miR-630 expression and serum AFP levels. **(A)** The expression of miR-630 in hepatocellular carcinoma tissues in patients with normal serum AFP (≤ 25 ng/ml; n=13) and abnormal serum AFP (>25 ng/ml; n=11) is shown. **(B)** The correlation analysis of miR-630 expression in hepatocellular carcinoma tissues and serum AFP levels is shown.

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