Down-Regulation of MiR-193a-3p Dictates Deterioration of HCC: A Clinical Real-Time qRT-PCR Study

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Although some recent reports have shown that the expression level of miR-193a varied in different cancers, its role in hepatocellular carcinoma (HCC) remains unidentified. The aim of the current study was to validate the relationship between miR-193a-3p and clinicopathological characteristics in HCC patients.

Material/Methods: Expression of miR-193a-3p in 95 HCC cases and their corresponding peritumoral tissues (PT) was examined by using quantitative reverse transcription polymerase chain reaction (qRT-PCR). miR-193a-3p expression and its correlation with a variety of clinicopathological features and patient recurrence were analyzed.

Results: The relative level of miR-193a-3p was 3.2028±1.1951 in PT, significantly higher than its expression in HCC tissues (1.5941±0.7079, $P<0.001$). The area under the curve of underexpression of miR-193a-3p was 0.906 to distinguish HCC from normal liver (95% CI: 0.864–0.948, $P<0.001$). Expression of miR-193a-3p was negatively correlated to metastasis (r=–0.371, $P=0.000$), TNM (r=–0.321, $P=0.002$), respectively. Additionally, the recurrence time was 50.271±2.631 months for the low miR-193a-3p level group and 60.132±3.626 months for the high miR-193a-3p level group. However, no significant difference between them was found (chi-square=0.354, $P=0.552$).

Conclusions: MiR-193a-3p may be a tumor-suppressive miRNA which is down-regulated in HCC tissues. It could be regarded as a predictor for the deterioration of HCC patients.

MeSH Keywords: Carcinoma, Hepatocellular • MicroRNAs • Neoplasm Metastasis • Neoplasm Staging • Reverse Transcriptase Polymerase Chain Reaction

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Background

Over the past decade the incidence of hepatocellular carcinoma (HCC) has doubled. In the Asia-Pacific region, 500 000 new cases occurred every year, with an estimated 360 000 deaths in Far East countries only [1]. China alone had more than 60% of the total HCC cases, most of which were due to chronic hepatitis B virus (HBV) infection [2–4]. In Japan, hepatitis C virus-(HCV-) related HCC made up 70% of all cases. In the US, where the 5-year survival rate remained under 12%, the incidence of HCC has tripled during the last 2 decades. In the European countries, because of the increased incidence of HCV, the occurrence of HCC has also increased [5,6]. Results from imageological examination, pathological biopsy, and serum AFP level are regarded as diagnostic criteria. Recently, the role of serum AFP level as an indicator for HCC has been shown to be less useful than previously assumed [7] and in most cases, the patients had late-stage HCC when diagnosed [8,9]. Only with early detection of the disease can we maximize clinical benefits while minimizing the toxicity and cost of nonsurgical treatments [10,11]. Therefore, there is a compelling need to find robust new diagnostic biomarkers.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that regulate the expression of protein-coding genes in a sequence-specific manner, including the genes controlling the DNA methylation state of the genome [12]. They can trigger the translational repression, deadenylation, and degradation of mRNA through binding to part of complementary sites within the 3'-untranslated region (UTR) of target messenger RNAs (mRNAs) [13]. Accumulating evidence shows that miRNAs controlled widely ranging physiological and pathological processes, including carcinogenesis and tumor progression [14]. Additionally, studies also found that miRNAs are involved in the invasion and metastasis of variety of cancers, including HCC [14,15]. Aberrant miRNA expression is also regarded as a promising biomarker for cancer detection.

Previous studies have shown that miR-193a repressed expression of certain oncogenic factors, including CDK6, c-kit, E2F6, and E-cadherin, which could enhance tumor invasiveness and angiogenesis [16–18]. Studies have also indicated that miR-193a exhibited significantly down-regulated expression in non-small cell lung cancer (NSCLC), and that they were associated with proliferation, metastasis, and apoptosis [19,20]. In epithelial ovarian cancer cells, miR-193a-3p plays a tumor-suppressive role [21], and in the neo-adjuvant chemotherapy (NACT) setting of ovarian cancer, miR-193a-5p is significantly over-expressed. In breast cancer, miR-193a-3p modulates the response of tumor cells to estrogen and anti-endocrine therapy, and up-regulated miR-193a-3p exaggerates the effects of estradiol and tamoxifen on estrogen receptor (ER)-positive breast cancer cells [22]. In colorectal cancer (CRC) patients, low

expression of miR-193a-3p is correlated with poor prognosis and lymph node metastasis [23]. As for HCC, a study based on HCC cell lines and *in vivo* study of nude mice showed that miR-193a-3p regulated the 5-fluorouracil (5-FU) resistance of HCC, whose transcription could be repressed by the hypermethylated promoter state [17]. Another study verified the molecular interaction between miR-193a-3p and urokinase-type plasminogen activator (uPA) mRNA target. The study evaluated the expression of miR-193a-3p in HCC tumoral tissues from 39 patients and concluded that miR-193a-3p was down-regulated in HCC compared to the peritumoral tissues (PT) [24]. However, no study has assessed the correlation of miR-193a-3p expression between HCC and matched PT along with its clinicopathological significance in HCC patients. Thus, in order to investigate the relationship between expression of miR-193a-3p and clinicopathological features of HCC, especially its metastasis and recurrence, we quantified miR-193a-3p expression in HCC tissues and their corresponding PT.

Material and Methods

Patients and tissue samples

There were 95 HCC patients in total recruited to the current study. Among them, 75 were males and 20 were females, the mean age was 52 years old (range 29–82 years old). All patients were admitted to the First Affiliated Hospital, Guangxi Medical University from March 2010 to December 2011. We retrospectively evaluated the formalin-fixed, paraffin-embedded (FFPE) cancer tissues and corresponding PT. We also collected their clinicopathological parameters such as age, sex, tumor differentiation, tumor diameter, number of tumor nodes, metastasis, TNM stages, status of portal vein tumor embolus, vaso-invasion, capsular infiltration, and cirrhosis. In addition, enzyme-linked immunosorbent assay (ELISA) was applied to measure the expression of AFP in sera and immunohistochemistry was used to detect the level of metadherin (MTDH), nm23, p53, p21, vascular endothelial growth factor (VEGF), and microvessel density (MVD) calculated with CD34 in cancer tissues. All these features are recorded in Table 1. The current study was approved by the Ethics Committee of First Affiliated Hospital, Guangxi Medical University, China. Informed consent was achieved from all patients participating in the study, conducted according to the Helsinki Declaration. All samples were re-examined by 2 independent pathologists (Fanghui Ren and Gang Chen).

qRT-PCR

Isolation and normalization of RNA were performed as described before [25–27]. Reverse transcription (RT) and qPCR kits were used to examine the expression of miR-193a-3p, as reported

Table 1. Relationship between the expression of miR-193a-3p and clinicopathological parameters in HCC.

Clinicopathological parameters		N	miR-193a-3p relevant expression($2^{-\Delta Cq}$)		
			Mean ±SD	t	P
Tissue	Adjacent liver	95	3.2028±1.1951	−11.288	<0.001
	HCC	95	1.5941±0.7079		
Age	<50	46	1.6802±0.7575	1.151	0.253
	≥50	49	1.5133±0.6555		
Gender	Male	75	1.5035±0.5747	−1.814	0.083
	Female	20	1.9340±1.0193		
Differentiation	High	6	1.6900±0.7780	F=0.063[a]	0.939
	Moderate	60	1.5820±0.7234		
	Poor	29	1.5993±0.6851		
Size	<5 cm	77	1.4006±0.3158	2.082	0.041
	≥5 cm	18	1.6394±0.7659		
Tumor nodes	Single	52	1.7544±0.8745	2.673	0.009
	Multiple	43	1.4002±0.3502		
Metastasis	−	46	1.9241±0.8428	4.803	0.000
	+	49	1.2843±0.3364		
Clinical TNM stage	I–II	22	2.2718±1.0485	3.866	0.001
	III–IV	73	1.3899±0.3886		
Portal vein tumor embolus	−	63	1.6910±0.8202	2.400	0.018
	+	32	1.4034±0.3428		
Vaso-invasion	−	59	1.7178±0.8066	2.535	0.013
	+	36	1.3914±0.4470		
Tumor capsular infiltration	With complete capsule	45	1.7291±0.7708	1.784	0.078
	Infiltration or no capsule	50	1.4726±0.6294		
HCV	−	63	1.6976±0.7932	2.420	0.017
	+	32	1.3903±0.4433		
HBV	−	17	1.5453±0.6738	−0.312	0.756
	+	78	1.6047±0.7189		
AFP	−	41	1.5978±0.6774	0.326[b]	0.745
	+	38	1.6511±0.7723		
Cirrhosis	−	50	1.6630±0.9080	−1.038	0.303
	+	45	1.5176±0.3755		
MTDH	−	38	1.6166±0.7713	0.996[c]	0.322
	+	51	1.4792±0.5295		

Table 1. Relationship between the expression of miR-193a-3p and clinicopathological parameters in HCC.

Clinicopathological parameters		N	miR-193a-3p relevant expression($2^{-\Delta Cq}$)		
			Mean ±SD	t	P
nm23	–	20	2.0550±1.0977	2.314	0.031
	+	75	1.4712±0.5045		
p53	–	40	1.7810±0.8596	2.092	0.041
	+	55	1.4582±0.5422		
p21	–	62	1.6216±0.7549	0.517	0.606
	+	33	1.5424±0.6177		
VEGF	–	25	1.8120±0.9747	1.431	0.163
	+	70	1.5163±0.5735		
Ki-67 LI	Low	47	1.7004±0.8314	1.451	0.151
	High	48	1.4900±0.5508		
MVD	Low	47	1.6670±0.8120	0.993	0.323
	High	48	1.5227±0.5887		

[a] ANOVA analysis was applied to assess the difference of miR-193a-3p among differentiation grading; [b] 16 cases missed the detection of AFP; [c] 6 cases lacked the measurement of MTDH. N – number; AFP – alpha fetal protein; MTDH – metadherin; VEGF – vascular endothelial growth factor; LI – labeling index; MVD – microvessel density.

previously [25–28]. A combinatorial reference of RUN6B and RUN48 was used for the detection of miR-193a-3p expression [29,30]. The primers for miR-193a-3p (Applied Biosystems Cat. No. 4427975-0000223: AACUGGCCUACAAAGUCCCAGU), RNU6B (Applied Biosystems Cat. No. 4427975-001093: CGCAA GGAUGACACGCAAAUUCGUGAAGCGUUCCAUAUUUUU) and RNU48 (Applied Biosystems Cat. No. 4427975-001006: GAU GACCCCAGGUAACUCUGAGUGUGUCGCUGAUGCCAUCAC CGCAGCGCUCUGACC) were in TaqMan® MicroRNA Assays (4427975, Applied Biosystems, Life Technologies, Grand Island, NY 14072, USA). TaqMan® MicroRNA Reverse Transcription Kit (4366596, Applied Biosystems, Life Technologies Grand Island, NY 14072 USA) also provided reverse primers, which were used in the RT process with a total volume of 10 µl. Applied Biosystems PCR7900 was used to perform the real-time qPCR for detection of miRNA. The expression of miR-193a-3p in the FFPE experiments was calculated with the formula $2^{-\Delta Cq}$ [25,26,29,30].

Statistical analysis

SPSS 20.0 (Munich, Germany) was used for the analysis of statistics. Student's t test was performed to investigate the significance of alteration between 2 groups. As we divided differentiation into 3 groups, 1-way analysis of variance (ANOVA) test was performed to distinguish its significance of alteration. Spearman correlation was used to study the relationship

between miR-193a-3p expression and clinicopathological parameters. Receiver operating characteristic (ROC) curve was drawn to test the effectiveness of miR-193a-3p when distinguishing HCC from PT. The relationship between miR-193a-3p and recurrence was analyzed with Kaplan-Meier (K-M) method. The log-rank test was performed to compare the recurrence time between different groups of miR-193a-3p. P-value less than 0.05 (2-tailed test) was statistically significant.

Results

Characteristics of the patients in current study

The clinicopathological parameters of the 95 HCC patients are enumerated in Table 1. All patients received no local ablative therapy, for example, percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), and no transcatheter arterial chemoembolization (TACE) or transarterial radioembolization (TARE) except liver resection. Tumor sizes varied from 1 to 11 cm with a mean size of 6.4 cm. The follow-up time ranged from 2.68 to 68 months (mean time: 33.13±1.71 months). Among the 70 HCC patients available to the follow-up, 11 were censored or dead at the end of follow-up and the rest had recurrent tumors.
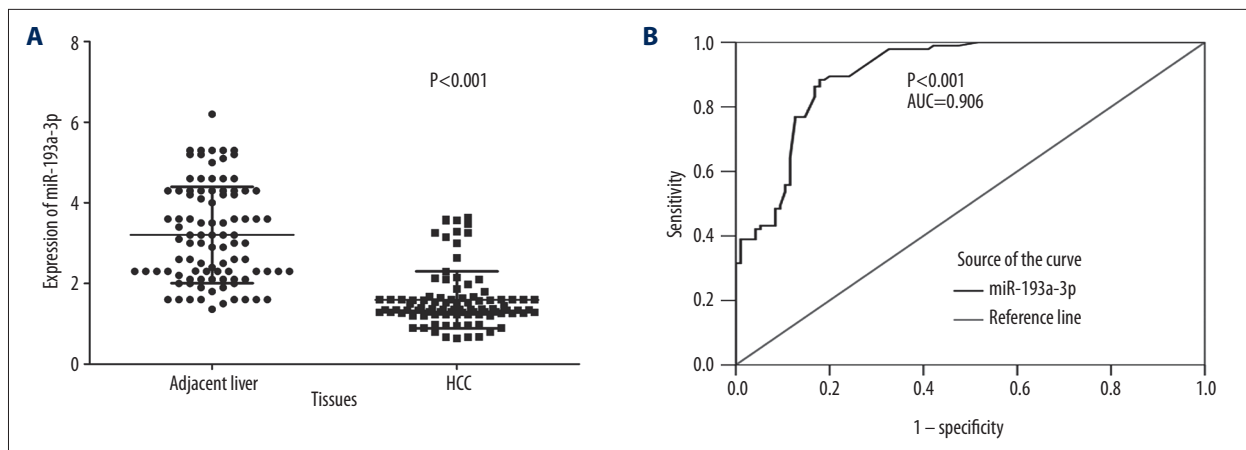
Figure 1. Expression of miR-193a-3p in adjacent PT and HCC tissues. Quantitative real-time RT-PCR was performed to detect the expression of miR-193a-3p. (**A**) The difference of relevant miR-193a-3p expression between adjacent PT and HCC tissues. (**B**) ROC curve of miR-193a-3p expression to distinguish HCC from PT. The area under curve (AUC) of miR-193a-3p was 0.906 (95% CI: 0.864-0.948, *P*<0.001).
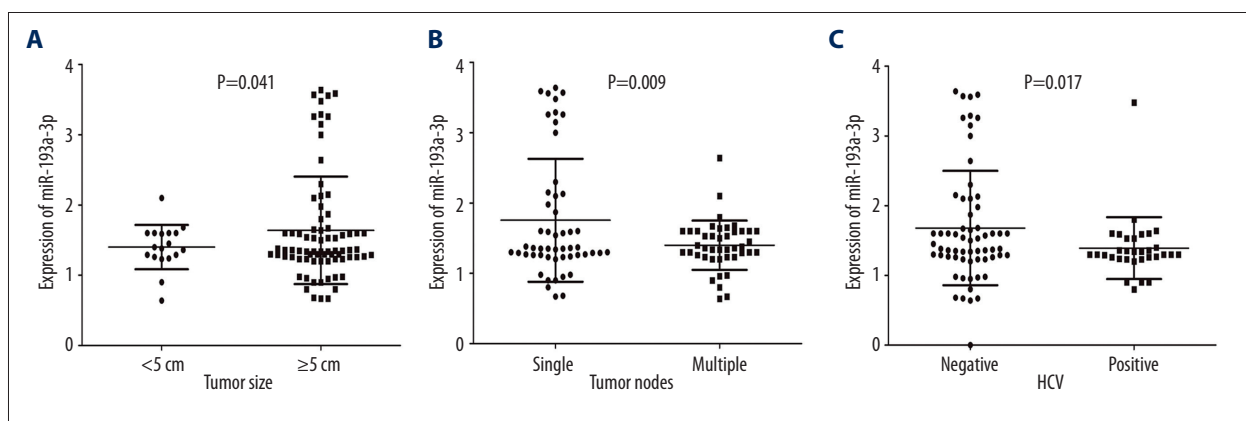
Figure 2. The relationship between miR-193a-3p and tumor size, tumor nodes, and HCV infection in HCC. (**A**) The difference of relevant miR-193a-3p expression between tumor smaller than 5cm and tumor larger than 5cm, *P*=0.041. (**B**) The difference of relevant miR-193a-3p expression between single tumor node and multiple tumor nodes, *P*=0.009. (**C**) The difference of relevant miR-193a-3p expression between negative infection and positive infection of HCV, *P*=0.017.

Diagnostic value of miR-193a-3p in HCC

A significant difference in miR-193a-3p expression level was found between HCC tissues and the relevant PT. The relative level of miR-193a-3p was 1.5941±0.7079 in HCC tissues, which was clearly lower than that of the PT (3.2028±1.1951, *P*<0.001, Figure 1A). In addition, we performed ROC curve to prove the diagnostic role of miR-193a-3p in HCC. The area under curve (AUC) of miR-193a-3p was 0.906 (95% CI: 0.864–0.948, *P*<0.001, Figure 1B).

The relationship between miR-193a-3p level and various HCC clinicopathological parameters

In HCC patients with tumor size was under 5 cm, their levels of miR-193a-3p (1.4006±0.3158) were clearly lower than those

with tumor size larger than 5 cm (1.6394±0.7659, *P*=0.041, Figure 2A). The relative level of miR-193a-3p in HCC patients with multiple tumor nodes (1.4002±0.3502) was prominently lower than those with single tumor nodes (1.7544±0.8745, *P*=0.009, Figure 2B). In association with HCV infection, HCC patients with positive infection (1.3903±0.4433) had greater down-expression of miR-193a-3p than those without infection (1.6976±0.7932, *P*=0.017, Figure 2C). Compared with those without metastasis (1.9241±0.8428), the expression of miR-193a-3p was reduced in HCC patients with metastasis (1.2843±0.3364, *P*<0.001, Figure 3A). ROC curve showed an AUC of 0.714 (95% CI: 0.612–0.817, *P*<0.001, Figure 3B) to predict the status of metastasis. When compared to early-stage patients (I and II, 2.2718±1.0485), the relative level of miR-193a-3p in those with advanced stages (III and IV, 1.3899±0.3886, *P*<0.001, Figure 3C) markedly decreased. The
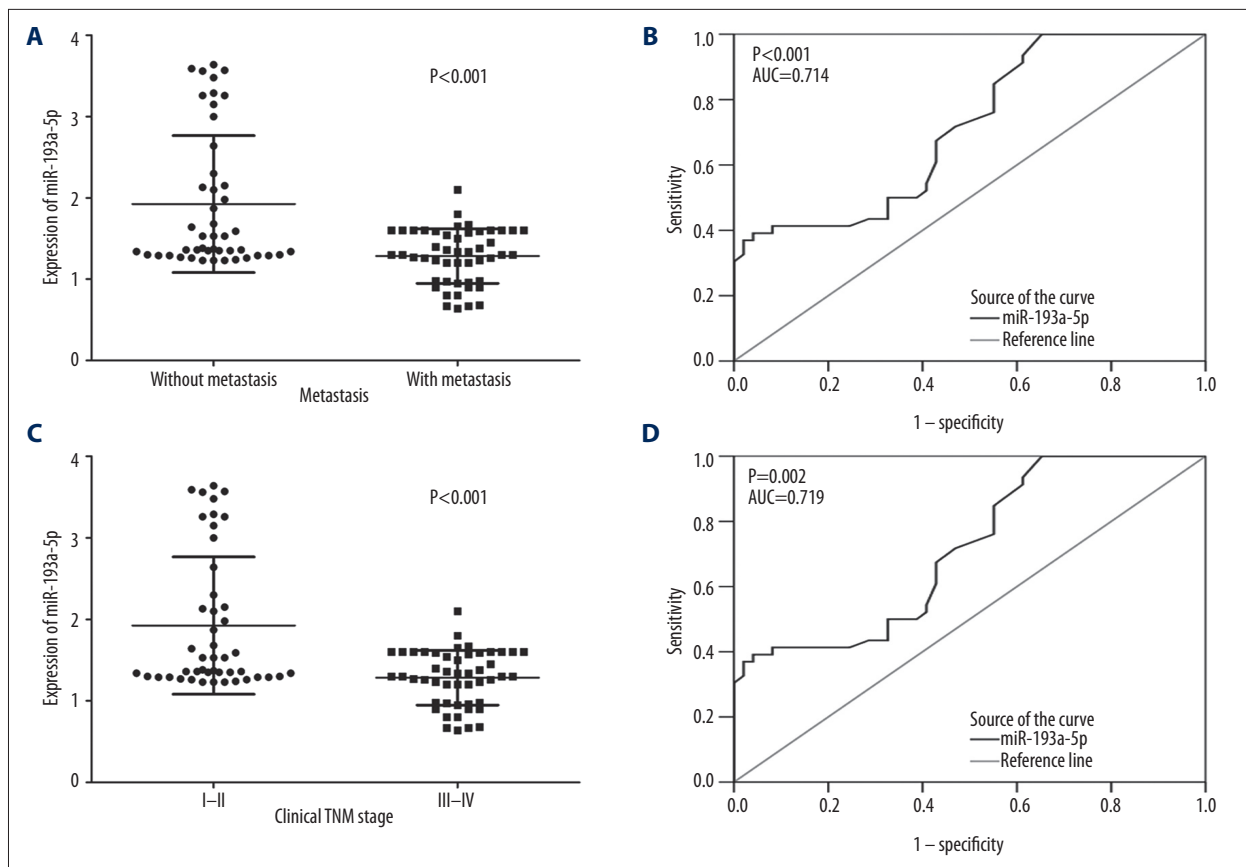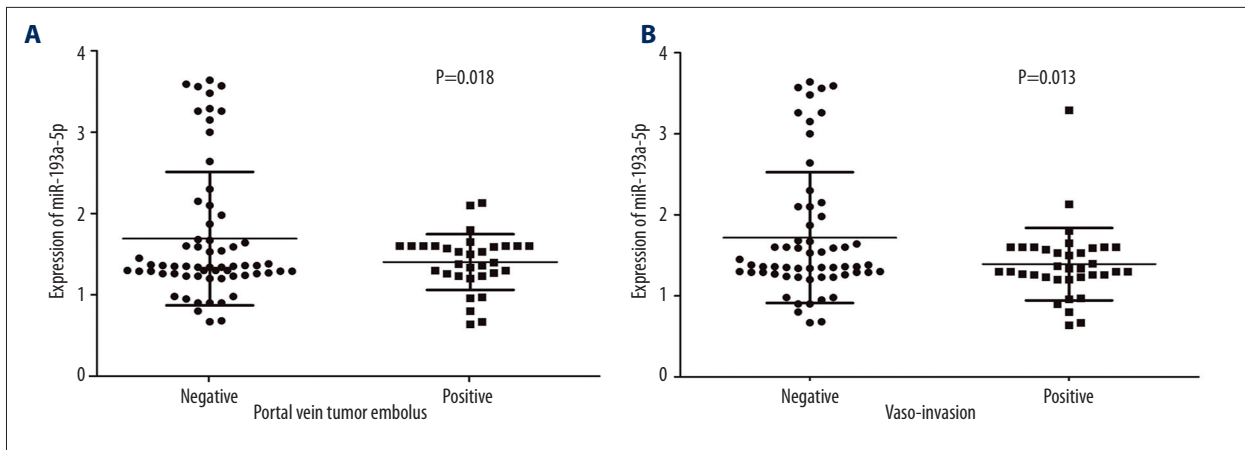
Figure 3. The relationship between miR-193a-3p and metastasis, clinical TNM stage in HCC. (**A**) The difference of relevant miR-193a-3p expression between patients without metastasis and with metastasis. *P*<0.001. (**B**) ROC curve of miR-193a-3p expression to distinguish whether there was metastasis for HCC patients. The area under curve (AUC) was 0.714 (95% CI: 0.612–0.817, *P*<0.001). (**C**) The difference of relevant miR-193a-3pexpression between early TNM stage and advanced TNM stage. *P*<0.001. (**D**) ROC curve of miR-193a-3p expression to distinguish early TNM stage from advanced TNM stage. The area under curve (AUC) was 0.719 (95% CI: 0.590-0.848, *P*=0.002).

AUC of ROC curve was 0.719 (95% CI: 0.590-0.848, *P*=0.002, Figure 3D) to evaluate the clinical TNM stage of HCC. In HCC patients who had portal vein tumor embolus, levels of miR-193a-3p (1.4034±0.3428) were prominently lower than those who had no embolus (1.6910±0.8202, *P*=0.018, Figure 4A). Additionally, the relative level of miR-193a-3p in HCC patients with vaso-invasion (1.3914±0.4470) was obviously lower than those without (1.7178±0.8066, *P*=0.013, Figure 4B). With respect to the correlation with the expression of nm23, HCC patients of nm23 positive (1.4712±0.5045) showed a noticeably lower level of miR-193a-3p than the negative one (2.0550±1.0977, *P*=0.031, Figure 5A). Moreover, miR-193a-3p in p53-positive HCC patients was down-regulated (1.4582±0.5422), compared with p53-negative HCC patients (1.7810±0.8596, *P*=0.041, Figure 5B). A further Spearman correlation test was performed and showed a negative correlation between miR-193a-3p and metastasis (r=−0.371, *P*<0.001), as well as between miR-193a-3p and clinical TNM stages (r=−0.321, *P*=0.002). However, no noteworthy

association was observed between miR-193a-3p level and other clinicopathological features.

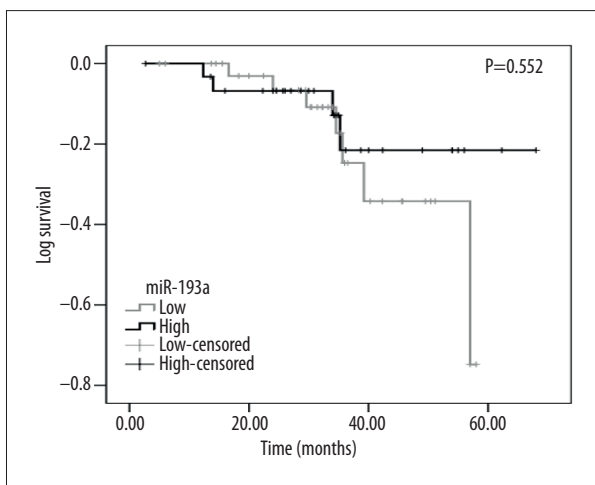### Role of miR-193a-3p expression in recurrence of HCC

Out of the 95 patients participating in our study, 70 remained available in the follow-up, with an overall recurrence time of 57.095±2.876 months. Among the 70 patients, 38 had low miR-193a-3p expression (lower than the median level of 1.36), while 32 had high miR-193a-3p expression. Concerning time to recurrence, low miR-193a-3p expression group was 50.271±2.631 months, shorter than that in the high expression group (60.132±3.626 months). However, no marked alteration of recurrence time was achieved between the low and high miR-193a-3p groups (chi-square=0.354, *P*=0.552, Figure 6).

Figure 4. The relationship between miR-193a-3p and portal vein tumor embolus and vaso-invasion in HCC. (**A**) The difference of relevant miR-193a-3p expression between negative and positive portal vein tumor embolus. *P*=0.018. (**B**) The difference of relevant miR-193a-3p expression between no vaso-invasion and positive vaso-invasion. *P*=0.013.
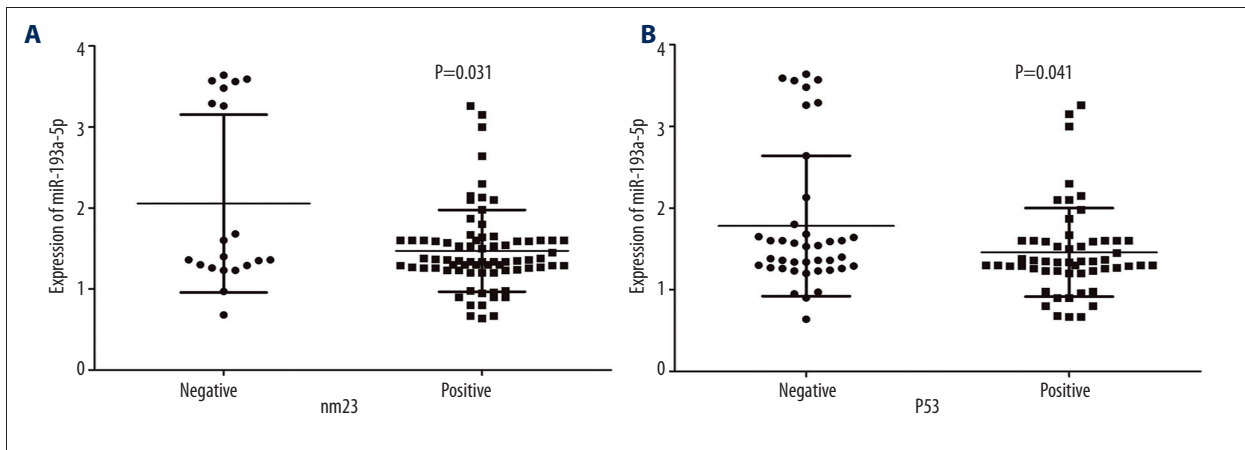
Figure 5. The relationship between miR-193a-3p and nm23, p53 in HCC. (**A**) The difference of relevant miR-193a-3p expression between negative and positive expression of nm23. *P*=0.031. (**B**) The difference of relevant miR-193a-3p expression between negative and positive expression of p53. *P*=0.041.

Figure 6. Recurrence K-M curve between high and low level of miR-193a-3p expression.

Discussion

Only 2 studies have explored the role of miR-193a-3p in HCC. One was carried out by Ma et al. [17], and a pair of HCC cell lines that differ in chemoresistance to 5-fluoro-2,4(1 h, 3 h) py-rimidinedione (5-FU) were selected to perform both genome-wide analysis and mechanistic studies. They found that the expression level of miR-193a-3p was low in 5-FU-sensitive HCC cell line HepG2, while it was high in all other 5-FU-resistant cell lines: PLC, FOCUS, Hep3B, and BEL-7402. They also selected nude mice for *in vivo* study to investigate the potential mechanism, and found that repression of the miR-193a-3p level by antagomir technologies inhibited tumor growth and sensitized SMMC-7721 cells to 5-FU. miR-193a-3p regulated the 5-FU resistance of HCC by targeting SRSF2 and E2F1. The other study was presented by Salvi et al. [24], who aimed to understand the co-treatment of HCC cells with the combination of a molecular

targeting drug (sorafenib) and miRNAs that target uPA. As a result, they validated that miR-193a-3p was a negative regulator of uPA in 2 HCC undifferentiated cell lines. Also, they evaluated the expression of miR-193a-3p in HCC tumoral tissues from 39 patients and found there was dysregulation of miR-193a-3p in tumoral tissues. However, the sample size was small (n=39) in the study of Salvi et al. [24]. In the present study, we examined the miR-193a-3p expression in 95 cases of HCC tissues and their corresponding PT. We found lower expression of miR-193a-3p in HCC tissues, which coincided exactly with the results of Salvi et al. [24]. The relevant level of miR-193a-3p was 1.5941±0.7079 in HCCs, which was significantly lower than that of the PT (3.2028±1.1951, $P<0.001$). The additional ROC curve demonstrated that miR-193a-3p had a valid diagnostic value for HCC, with the AUC of 0.906. Thus, we were strongly convinced that miR-193a-3p was a tumor-suppressive predictor in HCC.

No previous study has focussed on the relationship between expression of miR-193a-3p and clinicopathological parameters of HCC. We primarily hypothesized that miR-193a-3p expression was responsible for the deterioration of HCC for the following reasons: 1). There was a significant negative correlation between miR-193a-3p and metastasis as well as TNM stages and these 2 indexes indicate poor prognosis of HCC. The AUC of miR-193a-3p in metastasis and TNM was 0.714 and 0.719, respectively, which suggested its role in diagnosis and prognosis of speculation. 2). MiR-193a-3p was expressed at low levels in HCC patients whose tumors were smaller than 5 cm. It was also repressed in those patients with multiple tumor nodes compared to those with single node. 3). As HCC developed, there appeared portal vein tumor embolus and vaso-invasion, and miR-193a-3p was found to be down-regulated along with them. 4). In nm23-positive patients, level of miR-193a-3p decreased compared to nm23-negative patients, as is also true in p53-positive patients. MiR-193a-3p level was lower than that in p53-negative patients. Studies have already suggested that nm23 with pro-oncogenic potential was involved in hepatocarcinogenesis [31], and that its expression in tumor tissues was correlated with metastasis and survival in HCC, which may also be an indicator for prognosis [32]. Studies also showed that overexpression of p53 contributed to the hepatocarcinogenesis and more advanced stage of HCC [33]. As we found there was significant difference of miR-193a-3p expression in nm23 as well as p53-positive and -negative HCC patients in the current study, miR-193a-3p may be a pro-oncogenic potential indicator, as nm23 and p53 are in HCC.

From the K-M curve, we observed a tendency that the recurrence of patients with low miR-193a-3p level was shorter than those with a high level, although there was no significant difference. This suggested that miR-193a-3p could be a predictor of prognosis and survival. However, studies with larger sample sizes are needed to test the significant difference between miR-193a-3p and recurrence, as well as the relationship between miR-193a-3p and overall survival of HCC.

The 95 patients recruited into the current study received no additional treatment, and their tumor tissues were compared to their own paired PT, which was a guarantee of minimized individual differences and better comparability. The most noteworthy point is that we primarily identified relationship between miR-193a-3p expression and clinicopathological features, especially its relationship with metastasis and TNM stages.

However, the present study has a few limitations. Firstly, the sample size in our study was still small, which limited its corroborative role in drawing the conclusion of the impact of miR-193a-3p in patients with HCC. Secondly, we only used the method of qRT-PCR in our current study to evaluate the expression of miR-193a-3p, and fluorescence *in situ* hybridization (FISH) could be another option to detect the expression level of miRNA. Thirdly, we only analyzed the recurrence-free survival (RFS) of patients rather than overall survival (OS), which may not be sufficient to reflect the value of miR-193a-3p in predicting the prognosis of HCC. Fourthly, this study was limited to exploring the relationship between miR-193a-3p and its clinicopathological features in HCC tissues, not the correlation of miR-193a-3p with the expression profiling of its potential targeting genes. Furthermore, it would be better to identify its function *in vivo* and *in vitro* at a further step and discover new target genes of miR-193a-3p.

Conclusions

Along with previous studies, the current observations confirm that miR-193a-3p is a tumor-suppressive miRNA that plays an essential role in the tumorigenesis and deterioration of HCC. It could also be regarded as a predictor of prognosis. Cohorts with larger sample sizes and further *in vitro* and *in vivo* studies are needed define the relevant mechanism of miR-193a-3p in HCC.

Statement

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest

The authors have no declared conflicts of interest.

Ethical considerations

The current study was approved by the Ethics Committee of First Affiliated Hospital, Guangxi Medical University, China. Related research was conducted in accordance with the Helsinki Declaration.

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