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Original Article

Expression of miR-151 in hepatocellular carcinoma tissues and its clinicopathological significance

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Abstract: MicroRNAs (miRNAs) are small noncoding RNAs that can become dysregulated in cancer and may predict survival. The effect of microRNA-151 on outcome in hepatocellular carcinoma (HCC) after liver transplantation (LT) has not been reported. The identification of new biomarkers to predict prognosis of HCC after LT is an important clinical need. The aim of this study was to investigate the prognostic value of miR-151 in HCC patients following LT. We quantified miR-151 using real-time quantitative reverse transcription polymerase chain reaction in 82 paired cancerous tissues and para-cancerous normal liver tissues and investigated the relationships among miR-151 expression, clinicopathological parameters, and overall survival. Among 82 paired samples, 60 cancer tissues overexpressed miR-151 compared with matched normal liver tissues. Patients with microvascular invasion or multi-tumor nodules showed significantly higher miR-151 expression than those without these variables, and miR-151 expression was further increased in patients with post-LT HCC recurrence compared with those without recurrence. Patients with higher miR-151 expression showed significantly poorer overall survival than individuals with lower miR-151 levels. Multivariate analysis revealed that high miR-151 was an independent predictor of poor prognosis. Our results associate increased miR-151 expression with HCC recurrence and prognosis, and also suggest that miR-151 is an independent predictor of survival in HCC patients after LT and may serve as a potential biomarker for prognosis in these patients.

Keywords: microRNA-151, hepatocellular carcinoma, liver transplantation, prognosis, overall survival

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer [1, 2] and the third leading cause of cancer-related deaths worldwide. Liver transplantation (LT) is an effective therapeutic option for HCC. However, although LT provides a reasonable survival benefit for patients with HCC [3, 4] the long-term survival of patients following surgery remains unsatisfactory due to the high frequency of HCC recurrence.

Considering the limitations of imaging studies and the limited sensitivity of available laboratory analyses, identification of new prognostic markers may be useful in guiding post-LT surveillance. microRNAs are small, non-coding RNA gene products containing 20-24 nucleotides that play an important role in all biological processes through post-transcriptional regulation of protein-coding genes [5]. Recent studies indicate that expression profiling of microRNAs is a superior method for prognostication over mRNA profiling. Tumor microRNAs show a unique expression profile in HCC that may predict recurrence [6, 7]. Recently, miR-151 was found to be up-regulated in several tissues [8-11]. Moreover, miR-151 can promote HCC cell invasion and metastasis by targeting RhoGDIA [12]. However, to our knowledge no study has reported the association between miR-151 and prognosis with regard to post-LT HCC recurrence.

In the present study, we evaluated miR-151 expression levels in 82 paired cancerous tissues and adjacent non-cancerous hepatic tissues using quantitative reverse transcription polymerase chain reaction (RT-PCR) of formalin-fixed paraffin-embedded (FFPE) samples and found that miR-151 was significantly up-

Table 1. Correlation between miR-151 expression and clinicopathological characteristics of HCC patients following LT

Parameter	N	miR-151 relevant expression level		*p*-value
		Low	High	
Age	82	52.54±5.022	53.04±4.205	0.157[a]
Sex				
Male	73	36	37	1.000[c]
Female	9	5	4	
Etiology of liver disease				
HBV	74	39	35	0.264[c]
Others	8	2	6	
Cirrhosis				
Yes	79	39	40	1.000[c]
No	3	2	1	
Milan criteria				
In	42	22	20	0.659[b]
Out	40	19	21	
Histologic grade				
Well/Moderately	67	36	31	0.153[b]
Poorly	15	5	10	
Tumor stage				
I+II	65	35	30	0.173[b]
III	17	6	11	
Tumor size (cm)				
≤5	51	28	23	0.255[b]
>5	31	13	18	
Tumor nodes				
Multi	27	9	18	0.034[b]
Single	55	32	23	
Micro-vascular invasion				
Yes	18	5	13	0.033[b]
No	64	36	28	
Pre-LT serum AFP level				
>400 μg/L	31	14	17	0.494[b]
≤400 μg/L	51	27	24	
HCC recurrence	26/82	8/41	18/41	-
Overall survival	55/82	33/41	22/41	-

AFP alpha-fetoprotein, MELD model for end -stage liver disease, [a]Unpaired student's t test; [b]Chi-square test; [c]Fisher's exact test.

regulated in HCC tissues. We also investigated the association of miR-151 expression with biomarkers of survival of HCC patients following LT and clinical variables.

Materials and methods

Patients and tissue samples

From June 2009 to December 2013, 82 patients underwent LT for HCC at the Liver Transplantation Surgery Unit, Chinese Armed Police General Hospital, Beijing, China. We obtained follow-up data for these patients. Inclusion criteria included the "up-to-seven" transplantation criteria for HCC [13]. Patients with large liver vessel HCC invasions or extra-hepatic metastases and those who were younger than 18 years were excluded. Post-transplant monitoring of recurrence was performed by standard imaging and/or biopsy techniques. Human primary HCC and adjacent non-tumor liver tissues (>1 cm from the tumor) were collected from FFPE tissue blocks and confirmed histologically. Clinical information was collected from patient records (**Table 1**). Median recurrence-free period was 10 months for patients with HCC recurrence and 23 months for patients without recurrence. Prospective written consent was obtained in accordance with ethical guidelines.

Isolation of total RNA FFPE tissue

Total cellular RNA was isolated from 8 × 10-μm sections of FFPE tissues with efficient recovery of small RNA using the RecoverAll Total Nucleic Acid Isolation Kit (P/N, AM1975; Invitrogen, Shanghai, China) according to the manufacturer's instructions. RNA concentrations were determined spectrophotometrically. RNA quality was confirmed using a Fisher Scientific NanoDrop 2000 Spectrophotometer.

Quantitative real-time RT-PCR

Reverse transcription, was performed with 200 ng of total RNA and a specific stem-loop RT primer for miR-151 with U6 snRNA as an endog-

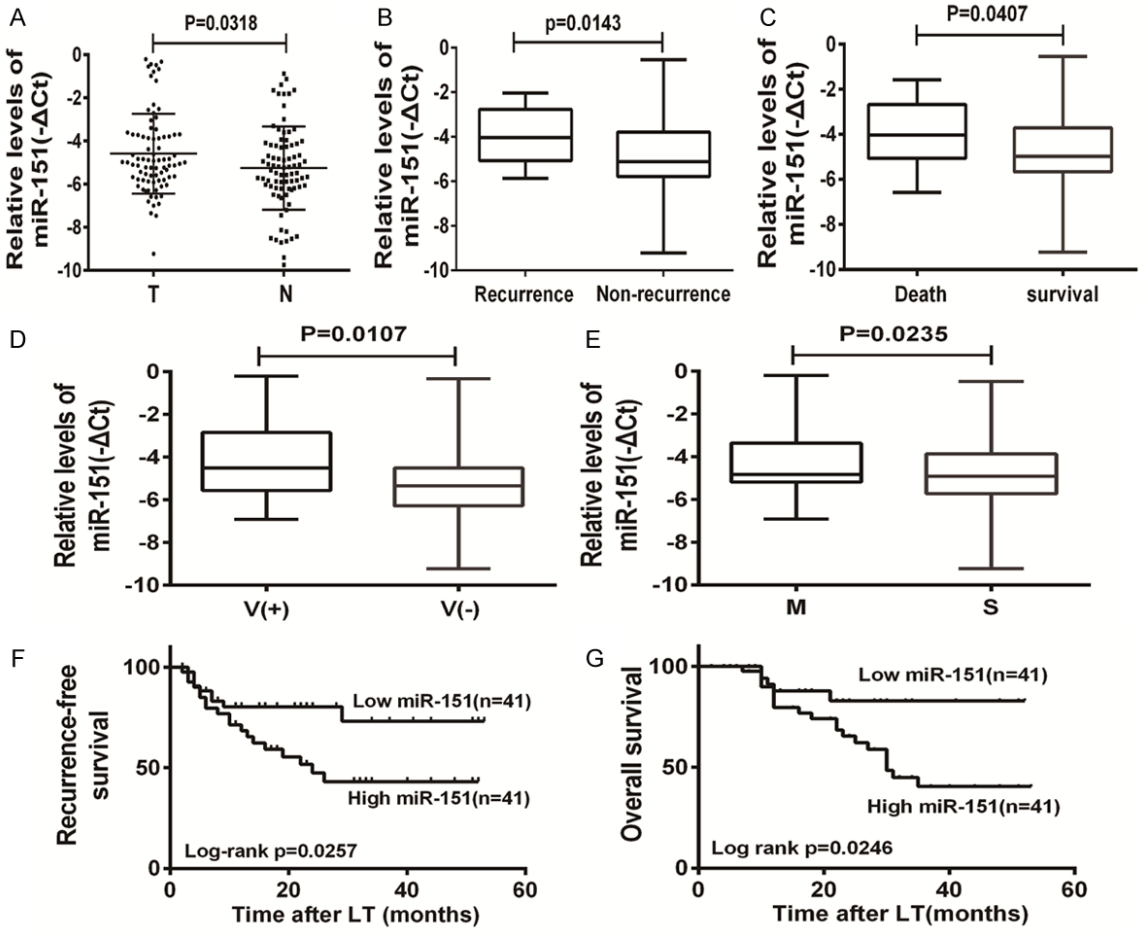

Figure 1. Upregulated of miR-151 in HCC is associated with tumor recurrence and poor prognosis following LT. A. The expression of miR-151 was examined in cancerous tissues (T) and their adjacent noncancerous hepatic tissues (N). B. The expression levels of miR-151 were further high-regulated in HCC samples of patients with tumor recurrence after LT. C. The expression levels of miR-151 were much higher in the patients who had died after LT than the patients who still survived. D and E. The expression of miR-151 were analyzed statistically in patients with micro-vascular invasion or without. v, micro-vascular invasion. m, multi tumor nodules. s, single tumor nodule. F and G. Kaplan-Meier analyse of survival curves for patients with high and low miR-151 expression levels.

enous control (Taqman miR-151/U6 snRNA assay; ID:000395 and ID:002324; Life Technologies, Foster City, CA, USA) using the TaqMan MicroRNA Reverse Transcription Kit (P/N 4366597; Life Technologies) and the following thermal conditions: 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. Quantitative RT-PCR amplification reactions were performed using TaqMan microRNA assays (P/N444-0887; Life Technologies) as described previously [14]; U6snRNA was used as an internal control to normalize and quantify miR-151 expression. Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. Quantitative PCR was quantified with an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Results represent mean ± standard deviation of three independent experiments. Two-sided Student's *t* test and the Mann-Whitney U test were applied to evaluate differences between groups. The chi-square test or the Fisher's exact test was used for analysis of categorical data. Multivariate Cox proportional hazard regression analysis was used to evaluate the contribution of independent factors to patient survival; only factors that were found to be significant in the univariate analysis were taken as covariates. All calculations were performed by GraphPad Prism 6 software (GraphPad Software, Inc., CA, USA). $P<0.05$ was considered statistically significant.

Table 2. Univariate and Multivariate Cox regression analyses of overall Survival in 82 HCC patients following LT

Parameter	Univariate analysis			Multivariable analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age	1.002	0.977-1.028	0.877	-	-	-
Gender	1.315	0.654-2.644	0.286	-	-	-
HBV infection	1.300	0.622-2.717	0.485	-	-	-
Cirrhosis	0.514	0.185-1.428	0.202	-	-	-
Tumor grade	1.390	0.790-2.446	0.253	-	-	-
Milan criteria (out)	1.269	1.873-3.423	0.028	0.906	0.516-1.571	0.514
AFP>400 (μg/L)	1.294	0.826-2.028	0.261			
Tumor diameter (>5 cm)	1.652	1.044-2.613	0.032	1.680	1.944-2.990	0.038*
Micro-vascular invasion	10.008	4.908-20.734	<0.001	5.568	2.912-10.649	<0.001*
miR-151 (high)	2.569	2.365-8.878	<0.001	3.177	2.630-9.744	0.002*

*Statistically significant difference.

Results

MiR-151 expression is higher in primary HCC than in matched adjacent non-cancerous hepatic tissues

To investigate the significance of miR-151 in HCC we examined miR-151 expression levels in a training set of 83 paired cancerous tissues and adjacent non-cancerous hepatic tissues using real-time RT-PCR. miR-151 expression was significantly up-regulated in 73.2% (60/82, *P*=0.0318) of primary HCC tissues (**Figure 1A**) compared with normal liver tissues. We next examined the correlation of miR-151 expression with clinicopathologic factors (**Table 1**). miR-151 expression was higher in patients with tumor recurrence after LT compared with those without recurrence after LT (*P*=0.0143; **Figure 1B**). A similar trend was seen in patients who died after LT compared with patients who survived (*P*=0.0407; **Figure 1C**). Moreover, patients with microvascular invasion or multi-tumor nodules expressed significantly higher miR-151 levels than those without these variables (*P*<0.05; **Figure 1D** and **1E**).

Increased miR-151 expression is associated with tumor malignancy

We analyzed the associations between miR-151 expression and clinicopathologic features and found that increased miR-151 expression in primary HCC was significantly associated with malignant behavior, such as microvascular invasion (*P*=0.033) and multiple tumor nodes (*P*=0.034) (**Table 1**); conversely, miR-151 expression in patients with microvascular invasion or multi-tumor nodules was significantly higher than in patients without these characteristics (*P*<0.05; **Figure 1D** and **1E**).

Increased miR-151 expression in HCC is associated with tumor recurrence and poor prognosis

To further explore the clinical relevance of miR-151, Kaplan-Meier and Univariate Cox proportional hazard regression analyses were performed. Kaplan-Meier analysis showed that increased miR-151 expression (i.e., > median level) correlated with shorter recurrence-free survival (RFS; *P*=0.0257; **Figure 1F**) and overall survival (OS; *P*=0.0246; **Figure 1G**) of HCC patients following LT. Univariate Cox regression analyses revealed that increased miR-151 expression was significantly associated with OS (*P*<0.001; hazard ratio [HR]: 2.569; 95% confidence interval [CI]: 2.365-8.878; **Table 2**).

MiR-151 independently predicts survival of post-LT HCC patients

To obtain insight into the predictive potential of miR-151 for survival we performed multivariate Cox proportional hazard regression analyses to test whether miR-151 expression was an independent prognostic factor associated with survival. Taking tumor size, tumor stage, histologic grade, Milan criteria, microvascular invasion, and miR-151 expression as covariates that were found to be significant in univariate analysis, univariate Cox regression for OS in the present patient cohort revealed that Milan

criteria, tumor diameter and microvascular invasion were strongly predictive of OS (**Table 2**). Multivariate Cox regression analysis showed that tumor diameter (HR=1.608, *P*=0.038), microvascular invasion (HR=5.568, *P*<0.001), and increased miR-151 expression (HR=3.177, *P*=0.002) were independently associated with poor survival (**Table 2**).

Discussion

Differential miR-151 expression between HCC tissues and non-cancerous liver tissues has only been reported in one previous study in which miR-151 expression was found to be up-regulated in HCC tissues compared with adjacent non-cancerous liver tissue samples (50 cases) using real-time RT-qPCR [12]. In the present study, increased expression of miR-151 in HCC tissues compared with corresponding adjacent liver tissues in the same patients was also observed. The results of our study, together with those of Ding [12], indicate that miR-151 plays a critical role in hepatocarcinogenesis, functioning as a oncogenic miRNA.

Post-transplant HCC recurrence can appear in two situations: when extra-hepatic metastasis was not detectable during the pre-transplant work-up, and as a consequence of circulating HCC cells engrafting and growing in a target organ during the peri-transplant period [15]. These two mechanisms may be associated with tumor cell aggressiveness. Expression profiling of microRNAs might be a more accurate method of classifying cancer subtypes than expression profiles of protein-coding genes [7, 16]. In particular, miR-151 is often over expressed in human tumors and promotes cancer cell invasion and metastasis [9-12]. In the present study, we showed that miR-151 expression was increased in HCC tissues. Moreover, increased miR-151 expression was associated with tumor malignancy; for example, increased miR-151 expression in HCC correlated with microvascular invasion and multiple tumor nodes. We further found that miR-151 was associated with survival and tumor recurrence in HCC patients who underwent LT. Our results indicated that HCC patients with higher expression levels of miR-151 had increased HCC recurrence and shorter survival following LT. To our best knowledge, our study is the first to correlate the expression of oncogenic miR-151 with clinical outcomes in HCC patients who underwent LT.

Mechanisms of miR-151 up regulation in various tumor tissues could be related to different target genes of miR-151 that have been reported in some cancers. Hsu KW *et al.* suggested that the Notch1 pathway and miR-151-5p interplay with p53 in a reciprocal regulation loop in the control of gastric carcinogenesis [17]. PCBP2 may facilitate promotion of glioma cell migration and invasion by miR-151-5p and miR-16 through mitigating the function of ARHGDIA [18]. The CCNE1 rs3218073 polymorphism located at a miRNA-151 binding site is associated with nasopharyngeal carcinoma (NPC) susceptibility and is correlated with NPC stage [19]. Our studies correlated increased miR-151 expression in HCC with microvascular invasion and multiple tumor nodes. Increased miRNA expression can contribute to tumor proliferation, survival, or invasiveness, depending on the target genes. However, the list of direct targets of miR-151 is far from complete. Moreover, the miR-151 targets in HCC are not fully understood and their identification and function warrant further study, as they may reveal new therapeutic targets or biomarkers for cancers.

Taken together with previous reports, the current observations strongly suggest that miR-151 acts as an oncogenic miRNA that plays a vital role in the tumorigenesis and progression of human HCC. MicroRNA-151 expression in HCC FFPE samples could be a prognostic biomarker for survival in HCC patients who undergo LT.

Disclosure of conflict of interest

None.

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References

[1] Lau WY, Lai EC. Hepatocellular carcinoma: current management and recent advances. Hepatobiliary Pancreat Dis Int 2008; 7: 237-257.

[2] de Villa V, Lo CM. Liver transplantation for hepatocellular carcinoma in Asia. Oncologist 2007; 12: 1321-1331.
[3] Befeler AS, Hayashi PH, Di Bisceglie AM. Liver transplantation for hepatocellular carcinoma. Gastroenterology 2005; 128: 1752-1764.
[4] El-Serag HB, Marrero JA, Rudolph L, Reddy KR. Diagnosis and treatment of hepatocellular carcinoma. Gastroenterology 2008; 134: 1752-1763.
[5] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
[6] Boyault S, Rickman DS, de Reynies A, Boyault S, Rickman DS, de Reyniès A, Balabaud C, Rebouissou S, Jeannot E, Hérault A, Saric J, Belghiti J, Franco D, Bioulac-Sage P, Laurent-Puig P, Zucman-Rossi J. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. Hepatology 2007; 45: 42-52.
[7] Reichl P, Mikulits W. Accuracy of novel diagnostic biomarkers for hepatocellular carcinoma: An update for clinicians (Review). Oncol Rep 2016; 36: 613-25.
[8] Sengupta S, den Boon JA, Chen IH, Newton MA, Stanhope SA, Cheng YJ, Chen CJ, Hildesheim A, Sugden B, Ahlquist P. MicroRNA 29c is down-regulated in nasopharyngeal carcinomas, up-regulating mRNAs encoding extracellular matrix proteins. Proc Natl Acad Sci U S A 2008; 105: 5874-5878.
[9] Guo Y, Chen Z, Zhang L, Zhou F, Shi S, Feng X, Li B, Meng X, Ma X, Luo M, Shao K, Li N, Qiu B, Mitchelson K, Cheng J, He J. Distinctive microRNA profiles relating to patient survival in esophageal squamous cell carcinoma. Cancer Res 2008; 68: 26-33.
[10] Rossi L, Bonmassar E, Faraoni I. Modification of miR gene expression pattern in human colon cancer cells following exposure to 5-fluorouracil in vitro. Pharmacol Res 2007; 56: 248-253.
[11] Mi S, Lu J, Sun M, Li Z, Zhang H, Neilly MB, Wang Y, Qian Z, Jin J, Zhang Y, Bohlander SK, Le Beau MM, Larson RA, Golub TR, Rowley JD, Chen J. MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia. Proc Natl Acad Sci U S A 2007; 104: 19971-19976.
[12] Ding J, Huang S, Wu S, Zhao Y, Liang L, Yan M, Ge C, Yao J, Chen T, Wan D, Wang H, Gu J, Yao M, Li J, Tu H, He X. Gain of miR-151 on chromosome 8q24.3 facilitates tumour cell migration and spreading through downregulating RhoGDIA. Nat Cell Biol 2010; 12: 390-399.
[13] D'Amico F, Schwartz M, Vitale A, Tabrizian P, Roayaie S, Thung S, Guido M, del Rio Martin J, Schiano T, Cillo U. Predicting recurrence after liver transplantation in patients with hepatocellular carcinoma exceeding the up-to-seven criteria. Liver Transpl 2009; 15: 1278-1287.
[14] Varkonyi-Gasic E, Hellens RP. Quantitative stem-loop RT-PCR for detection of microRNAs. Methods Mol Biol 2011; 744: 145-157.
[15] Toso C, Mentha G, Majno P. Liver transplantation for hepatocellular carcinoma: five steps to prevent recurrence. Am J Transplant 2011; 11: 2031-2035.
[16] Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857-866.
[17] Hsu KW, Fang WL, Huang KH, Huang TT, Lee HC, Hsieh RH, Chi CW, Yeh TS. Notch1 pathway-mediated microRNA-151-5p promotes gastric cancer progression. Oncotarget 2016; 7: 38036-38051.
[18] Lin X, Yang B, Liu W, Tan X, Wu F, Hu P, Jiang T, Bao Z, Yuan J, Qiang B, Peng X, Han W. Interplay between PCBP2 and miRNA modulates ARHGDIA expression and function in glioma migration and invasion. Oncotarget 2016; 7: 19483-98.
[19] Liu Y, Cai H, Liu J, Fan H, Wang Z, Wang Q, Shao M, Sun X, Diao J, Liu Y, Shi Y, Fan Q. A miR-151 binding site polymorphism in the 3'-untranslated region of the cyclin E1 gene associated with nasopharyngeal carcinoma. Biochem Biophys Res Commun 2013; 432: 660-665.