

Original Article

High expression of myofibrillogenesis regulator-1 predicts poor prognosis for patients with hepatocellular carcinoma after curative hepatectomy

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Received September 9, 2015; Accepted October 22, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Myofibrillogenesis regulator (MR-1) is overexpressed in human cancer cells and plays an essential role in cancer cell growth. However, its role in hepatocellular carcinoma (HCC) has not yet been explored. The aim of this study was to investigate the association of MR-1 expression with clinicopathologic features and prognosis in patients with HCC. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to detect MR-1 mRNA levels in tissues samples from 120 HCC patients. Results showed that MR-1 expression was significantly higher in HCC tissues when compared with matched adjacent normal tissues ($P=0.004$). In HCC cancerous tissues, it was also significantly associated with tumor size ($P=0.024$) and serum AFP level ($P=0.003$). Moreover, Kaplan-Meier analysis showed that HCC patients with high MR-1 expression had shorter overall survival time than those with low MR-1 expression ($P=0.009$). When analyzed with a multivariate Cox regression model, MR-1 was identified as an independent prognostic factor for overall survival. Furthermore, when combined with serum AFP level, the median survival time significantly differed between patients with baseline high serum AFP and high MR-1 expression levels and those with normal AFP and low MR-1 levels ($P=0.007$). Taken together, our results suggest that high expression of MR-1 is involved in HCC progression and could be a novel biomarker of poor prognosis in patients with HCC.

Keywords: Myofibrillogenesis regulator-1, hepatocellular carcinoma, prognosis, overall survival

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related death in the world. According to the International Agency for Research on Cancer, more of an estimated 748,300 new liver cancer cases and 695,900 cancer deaths occurred worldwide every year [1]. Despite the improvement of surgical techniques and perioperative management as well as the development of non-surgical treatments such as radiofrequency ablation (RFA) or transarterial chemoembolization (TACE), HCC prognosis remained poor because of advanced tumor stage accompanied by chronic liver disease (CLD) at diagnosis [2]. The identification of biomarkers correlating with the outcome of patients with HCC may help determine the prognosis, identify patients most like-

ly to benefit from specific treatments, and therefore could guide clinicians in designing personalized treatment strategies [3].

Myofibrillogenesis regulator-1 (MR-1), which is mapped to 2q35, was first cloned from a human skeletal muscle cDNA library using polymerase chain reaction (PCR) and rapid amplification of cDNA ends [4]. MR-1 is composed of three distinct exons, in which exon 3 is unique when compared with other two genes, and encodes a protein of 142 amino acids with a hydrophobic transmembrane structure from 75 to 92 amino acids [5-8]. The transcription level of MR-1 in human tissues is especially high in myocardium and skeletal muscles as revealed by Northern blot and serial analysis of gene expression [5]. Overexpression of MR-1 could promote cancer cell proliferation and migration in human hepatoma G2 (HepG2) cells [9]. MR-1 might promote

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cancer cell proliferation by binding to specific proteins, such as eukaryotic initiation factor 3 that is highly correlated with tumor cell growth and invasion regulation [10]. Also, overexpression of MR-1 can activate the nuclear factor κ B signaling pathway, which is correlated with a wide variety of diseases, including cancer, inflammation, and autoimmune diseases [11].

Taking all the evidences listed above into account, we hypothesized that MR-1 may take part in the development and progression of HCC. On the basis of these studies, we used real-time quantitative reverse transcriptase PCR (qRT-PCR) to examine the expression of MR-1 in HCC samples and adjacent normal tissues. Our study was the first attempt to investigate the relationship between MR-1 expression and prognosis of HCC patients with complete clinical and follow-up data. We evaluated the possible associations between MR-1 mRNA expression and clinicopathological characteristics. We also analyzed MR-1 mRNA expression and studied the relationship between MR-1 expression and overall survival.

Materials and methods

Patients

Between August 2009 and April 2011, 120 consecutive patients with HCC who underwent surgical resection as initial treatment at Department of Hepatobiliary Surgery in Chinese PLA General Hospital were enrolled in this study. HCC was reconfirmed in all patients on the basis of the American Association for the Study of Liver Diseases practice guidelines. The study inclusion criterion was early-stage HCC classified as Barcelona Clinic Liver Cancer (BCLC) stage 0 or A. The exclusion criteria were extrahepatic metastasis, Child-Pugh class C, and concurrent presence of another primary liver cancer (such as fibrolamellar HCC or cholangiocarcinoma) or other types of cancers. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by Institutional Review Board of Chinese PLA General Hospital. Written informed consent was obtained from all of the patients.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR was performed to detect the expression levels of MR-1 in HCC tissues and matched adjacent normal tissues. Total RNA was extract-

ed from tissue samples of 120 pairs of HCC and adjacent normal tissues using Trizol (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. RNU6B was used as internal control. The specific cDNA of MR-1 and RNU6B were synthesized from total RNA using gene-specific primers according to the TaqMan MicroRNA assays protocol (Applied Biosystems, Foster City, CA, USA). Reverse transcriptase reactions contained 10 ng of total RNAs, 50 nmol/l stem-loop RT primer, 1 \times RT buffer, 0.25 mmol/l each of deoxynucleotide triphosphate (dNTP), 3.33 U/ μ l MultiScribe reverse transcriptase, and 0.25 U/ μ l RNase Inhibitor. The 20 μ l reaction volumes were incubated in Bio-Rad i-Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a 96-well plate for 30 min at 15°C, 30 min at 40°C, 5 min at 85°C, and then held at 4°C. Real-time PCR was performed using an Applied Biosystems 7500 real-time PCR system. The reaction mixture (10 μ l total volume per well) included 2 ng cDNA, 1 \times TaqMan Universal PCR master mix, and 1 μ l of primers and probe mix of the TaqMan MicroRNA Assays. Relative quantification of target miRNA expression was evaluated using the comparative cycle threshold (CT) method. The raw data were presented as the relative quantity of target miRNA, normalized with respect to RNU6B. Each sample was examined in triplicate.

Follow-up

Patients were prospectively followed after surgery according to a formulated schedule. Follow-up information of all patients was updated every 3 months for the first 2 years, every 6 months for the next 3 years, and then every year thereafter. The follow-up time ranged from 7 months to 81 months (median, 44 months). At reference date (April 30, 2015), patients still alive were censored at their last consultation, and patients who died were censored at their death date. Overall survival time was calculated from the date of the initial surgical operation to death.

Statistical analysis

The MR-1 expression level was expressed as mean \pm standard deviation (SD). Associations between MR-1 expression level in HCC and clinicopathological features were determined using the χ^2 -test. The Kaplan-Meier method was used to estimate survival rates, and the log-rank test was used to assess survival differences

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Table 1. Association between MR-1 expression and clinicopathological features of HCC

Variables	All patients (n=120)	High MR-1 level (n=54)	Low MR-1 level (n=66)	P value
Male sex, n (%)	78 (65.0%)	36 (66.7%)	42 (63.6%)	0.762
Age, yr ± SD	57.2±10.4	58.3±11.7	55.9±9.3	0.694
Cirrhosis etiology (n)				
HBV/HCV/alcohol/other	99/8/10/3	45/3/5/1	54/5/5/2	0.173
Child-Pugh A/B (n)	105/15	48/6	57/9	0.548
Bilirubin, µmol/L, mean ± SD	19.2±10.4	18.1±9.8	20.3±11.3	0.664
Albumin, g/L, mean ± SD	38.8±5.8	39.7±6.5	37.8±5.6	0.459
Platelets, ×1000/ml	134.5±48.1	138.2±51.4	130.2±45.6	0.104
HCC median size, cm	3.7±0.9	4.9±1.2	3.1±0.8	0.024
HCC<3	36 (30.0%)	10 (18.5%)	26 (39.4%)	
HCC≥3	84 (70.0%)	44 (81.5%)	40 (60.6%)	
Serum AFP levels, ng/ml, mean ± SD	74.5±220.5	104.5±297.4	55.2±176.6	0.003
Edmondson grade III or IV, n (%)	29 (24.2%)	14 (25.9%)	15 (22.7%)	0.438

AFP α-fetoprotein, HBV hepatitis B virus, HCV hepatitis C virus, HCC Hepatocellular Carcinoma, MR-1 myofibrillogenesis regulator-1, SD standard deviation.

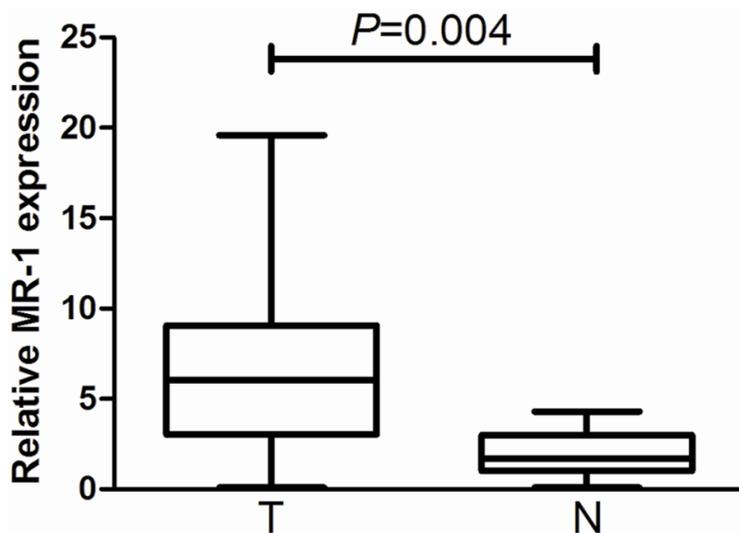


Figure 1. Relative expression of MR-1 to RNU6B in 120 pairs of HCC tissues (T) and adjacent normal tissues (N). The horizontal line inside the box is the mean, and the box represents the lower and upper quartiles. The whiskers indicate the minimum and maximum data values.

between groups. The Cox proportional hazards model for multivariate survival analysis was used to assess predictors related to overall survival. All statistical analyses were performed using SPSS software (SPSS 19.0, Chicago, IL, USA) and $P < 0.05$ was considered statistically significant.

Results

One hundred and twenty consecutive patients with BCLC O/A HCC who underwent curative

surgical resection were selected. The baseline characteristics of patients are summarized in **Table 1**. Patients were mainly males (65.0%) with compensated Child-Pugh A hepatitis B-related cirrhosis and single HCC (median diameter 37 mm). Fifty-five patients (45.8%) had normal serum AFP values (< 20 ng/ml) and 29 patients (24.2%) had high Edmondson grade (grade III or IV). The median MR-1 expression level was 5.7 (range 0.1-19.6) in HCC tissues, while it was significantly lower in matched adjacent normal tissues (median MR-1 expression level: 1.3; range 0.1-4.3; $P = 0.004$) (**Figure 1**). By adopting cut-off value according to the median MR-1 level in HCC tissues, patients were sorted into

two categories: 54 patients with high MR-1 levels and 66 patients with low MR-1 levels. As shown in **Table 1**, high MR-1 expression level was associated with larger tumor size ($P = 0.024$) and higher serum AFP level ($P = 0.003$).

During the follow-up period, 49 of the 120 patients (40.8%) died (30 patients with high MR-1 levels and 19 with low MR-1 levels). The median survival time was 56 months with significant differences between high MR-1 level and low MR-1 level (62 months vs. 49 months;

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Table 2. Univariate and multivariate analyses of prognostic factors in patients with HCC

Variables	Univariable <i>P</i> -value (log-rank test)	Multivariable	
		HR (95% CI)	<i>P</i> -value
Gender			
Male vs. Female	0.647		
Age at diagnosis (years)			
<50 vs. ≥50	0.481		
Tumor size (cm)			
<3 vs. ≥3	0.075		
Serum bilirubin (μmol/L)			
<17.1 vs. ≥17.1	0.236		
Serum albumin (g/L)			
<35 vs. ≥35	0.436		
Platelets (×1000/ml)			
<100 vs. ≥100	0.227		
Serum AFP levels (ng/ml)			
≥20 vs. <20	0.016	1.27 (1.09-1.45)	0.037
Edmondson grade			
III/IV vs. I/II	0.032	1.12 (0.97-1.28)	0.056
MR-1 expression			
High vs. Low	0.008	1.38 (1.20-1.59)	0.015

AFP α-fetoprotein, HCC Hepatocellular Carcinoma, HR hazard ratio, CI confidence interval, MR-1 myofibrillogenesis regulator-1.

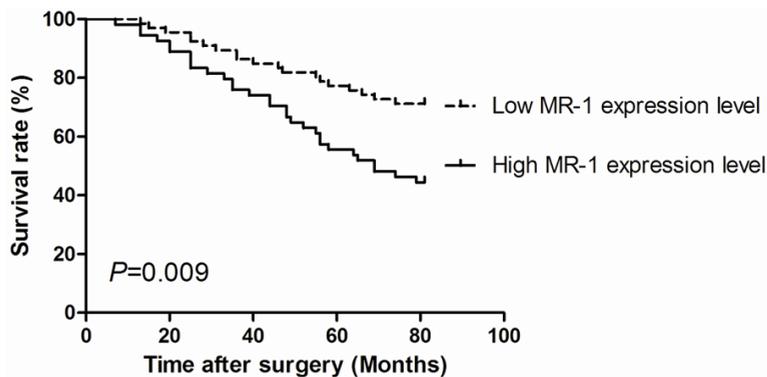


Figure 2. Kaplan-Meier estimates of overall survival between low and high MR-1 mRNA expression levels.

$P=0.009$) (**Figure 2**). Cut-off values for each of the clinical and pathological factors including MR-1 expression level were selected, and univariate analysis was carried out to identify those factors significantly associated with overall survival. The results showed that high serum AFP level, high Edmondson grade (grade III or IV), and high MR-1 level were associated with shorter overall survival time with a P value <0.05 and were entered into the multivariate

analysis. In the multivariate analysis, high serum AFP level (HR=1.27; 95% CI=1.09-1.45; $P=0.037$) and high MR-1 level (HR=1.38; 95% CI=1.20-1.59; $P=0.015$) were both independent predictive factors for overall survival (**Table 2**).

Figure 3 shows overall survival according to tumor expression of MR-1 combined with serum AFP level. The median survival time differed significantly among the 4 categories. In 31 patients with high serum AFP and high MR-1 expression levels, only 32.3% survived during the follow-up period after surgery, while survival rate reached 72.2% in 36 patients with baseline normal AFP and low MR-1 levels ($P=0.007$).

Discussion

It has been reported that MR-1 participates in tumor promotion in several types of human cancers, including ovarian cancer, gastric cancer, and pancreatic ductal adenocarcinoma [4, 12, 13]. However, no report is available regarding MR-1 expression in HCC tissues except that one study examined MR-1 expression in human hepatoma HepG2 cells [9]. They found that overexpression of MR-1 was associated proliferation and migration of human hepatoma HepG2 cells. To explore

the vital role of MR-1 in the tumorigenesis and progression of HCC, we examined expression patterns of MR-1 in HCC tissues and analyzed the relationship between MR-1 expression and clinicopathological factors of HCC. Our data revealed that MR-1 was significantly overexpressed in HCC tissues and its expression was correlated with tumor size and serum AFP level. More importantly, we also demonstrated that the expression of MR-1 was an independent

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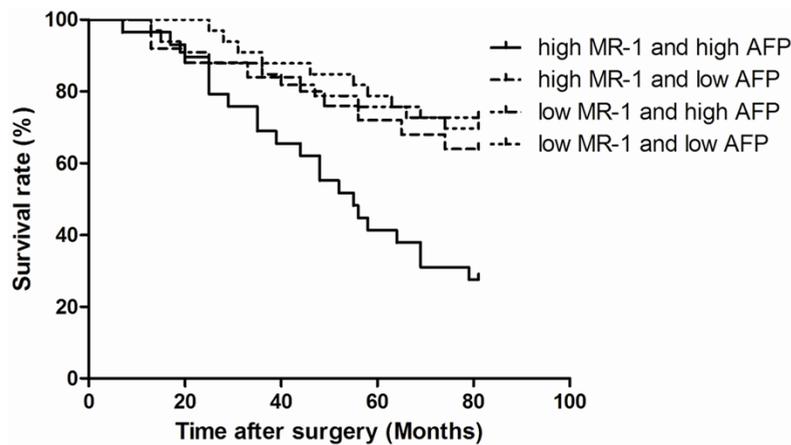


Figure 3. Kaplan-Meier estimates of overall survival according to the combined expression of MR-1 by HCC tissues and serum AFP level.

prognostic predictor for overall survival, suggesting that MR-1 is a potential tumor biomarker for HCC.

In the present study, we also showed that MR-1 expression could have an additional prognostic value to AFP levels. Indeed, the classification of patients according to the tumor expression of MR-1 combined with serum AFP level led to the identification of 4 groups with significantly different overall survival rates varying from 32.3% in patients with baseline high AFP and high MR-1 levels to 72.2% in patients with baseline high AFP and low miR-34a levels ($P=0.007$). These results could improve decision in the HCC therapeutic area. Stratification according to baseline serum AFP level and tumor MR-1 expression would allow a better selection of adequate candidates for liver transplantation following surgery, restricted to patients with baseline normal serum AFP and low MR-1 levels and would be helpful for designing clinical trials for HCC, aimed at assessing the benefit of adjuvant therapy in patients with unfavorable prognostic factors (i.e., baseline elevated serum AFP and/or high MR-1 expression in HCC tissues). However, to be included into guidelines of clinical management of early HCC, external validation studies are needed.

The mechanism by which MR-1 promotes tumorigenesis and cancer progression has not been well elucidated. The interaction of MR-1 with sarcomeric structural proteins involved in muscle contraction and its presence in human myocardial myofibrils indicate that MR-1 could regulate contractile proteins in the myocardium and might be associated with cardiac hypertro-

phy [4]. Myosin light chain -2 (MLC-2) plays an important role in cell migration from solid cancers and its dephosphorylation could induce apoptosis [14]. A study showed that MLC-2 may regulate cell proliferation and migration by interacting with MR-1 [15]. Knockdown of MR-1 expression in human hepatoma HepG2 cells inhibits cell migration and proliferation both in vitro and in vivo. The mechanism underlying this action is that MR-1 induces MLC-2 activation, subsequently stimu-

lates stress fiber formation, and indirectly activates the focal adhesion kinase (FAK)/Akt signaling pathway to promote cell migration and proliferation [8, 16]. Further studies are needed to define the molecular mechanisms that govern the potential role of MR-1 expression in HCC progression, clarify whether MR-1 is an early diagnostic marker for HCC and to assess its full therapeutic potential.

In conclusion, our results suggest that high expression of MR-1 is involved in HCC progression and could be a novel biomarker of poor prognosis in patients with HCC.

Acknowledgements

This work was supported by a grant from the National Science Foundation of China (No. 81340637).

Disclosure of conflict of interest

None.

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