Original Article
Expression of protein TARBP1 in human hepatocellular carcinoma and its prognostic significance

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Abstract: Objective: The objective of this study was to analyze the expression of TARBP1 and its clinical significance in hepatocellular carcinoma (HCC). Materials and Methods: 90 patients with primary hepatocellular carcinoma were included in this study. The tumor and paired adjacent non-tumor tissues were collected. TARBP1 expression was assessed by quantitative real-time polymerase chain reaction and immunohistochemistry. Associations of TARBP1 expression with the clinicopathological features were analyzed, and prognosis of HCC patients was evaluated. Results: The result show the expression of TARBP1 mRNA in liver cancer tissues were higher than in the adjacent normal liver tissues in 10 paired samples (P=0.0015). Compared with adjacent normal liver tissues, overexpression of TARBP1 was detected in 61.1% (55/90) HCC patients. TARBP1 expression was associated with the AJCC tumor stage (P=0.004) and clinical stage (P=0.005), and decreased overall survival (P=0.002). In multivariate analysis, TARBP1 expression was an independent prognostic factor for overall survival (Hazard ratio [HR]=2.773, 95% confidence interval [CI] 1.542-4.985; P=0.019). Conclusions: TARBP1 is up-regulated in HCC, and the expression of TARBP1 was associated with the pathological grading and clinical stage. TARBP1 maybe is an independent prognostic marker of HCC patients.

Keywords: Hepatocellular carcinoma-TARBP1- prognosis

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide, with the incidence on the rise [1]. Due to the high rate of recurrence and low detection rate at the curable stages, liver cancer has a poor prognosis. Statistics indicate that following hepatectomy, recurrence rates are high (approximately 50% at 3 years) [2], the survival rate of patients is 30-40% at five years [3]. In china, HCC is the second leading cause of cancer death, which accounts for about 50% of all HCC deaths worldwide [4-6]. In the United States, the incidence has approximately doubled in the past three decades [7]. The survival of HCC patients after resection remains poor, mainly attributing to frequent metastases and recurrence [8]. And one of the most important reason is lack of a universal HCC prognostic staging system [9]. Therefore, a comprehensive knowledge of the pathogenesis of liver cancer is urgently required and guide therapeutic options.

The TAR (transactivation response) RNA binding protein, TARBP1, is a cellular double-stranded RNA (dsRNA) binding protein that has been shown to promote the replication of human immunodeficiency virus types 1 and 2 (HIV-1 and -2) [10]. TARBP1 has more recently been found to be a constituent of the RNA-induced silencing complex (RISC) serving as a Dicer co-factor in the processing of the ~70 nucleotide pre-microRNAs (miRNAs) to 21-25 nucleotide mature miRNAs [11]. Recently, TARBP1 truncating mutations were found in human cancers with microsatellite instability [12]. Knockdown of TARBP1 can reduce the accumulation of hepatitis C virus RNA [13], and TARBP1 has been proposed as a target for antiviral therapies [10, 14, 15]. In this study, we sought to evaluate the expression of TARBP1 and its associated clinical significance in HCC.

Table 1. Patient characteristics and clinicopathologic correlation of TARBP1 expression levels

Characteristics	Total	TARBP1		P value
		Positive	Negative	
Gender				0.731
Male	81	50	31	
Female	9	5	4	
Age (years)				0.189
≥60	22	11	11	
<60	67	44	23	
Hepatitis				0.641
HBV/HCV	4	2	2	
Negative	86	53	33	
Live cirrhosis				0.708
Presence	33	21	12	
Absence	57	34	23	
Tumor size (cm)				0.318
≥5	53	35	18	
<5	36	20	16	
AJCC tumor stage				0.004
I	9	3	6	
II	48	25	23	
III	33	27	6	
Clinical stage				0.005
1	11	3	8	
2	29	15	14	
3	42	32	10	

HBV, hepatitis B virus; HCV, hepatitis C virus; AJCC, American Joint Committee on Cancer.

Materials and methods

Patients and specimens

The ethics committee at the First Affiliated Hospital of Guangzhou Medical University approved this study, and all the patients gave written informed consent on the use of clinical specimens for medical research. 90 HCC patients undergoing curative resection at our institution between January 2007 and November 2009 were included in this study. The demographic features and clinicopathologic data were detailed in **Table 1**. The median age of patients was 49 years (range 27-80 years), and the median tumorsize was 5.4 cm (range 1.5-17.1 cm). All the patients were diagnosed with primary HCC, and 4 (4.4%) patients were associated with chronic viral hepatitis (HBV 3 patients and HCV 1 patients). None of patients received any type of neoadjuvant therapy, and all underwent curative surgery. Both tumor and adjacent non-tumor tissue (the adjacent non-tumor tissue was defined as at least 1-cm distance from the tumor edge) were processed immediately after operation. TARBP1 expression was evaluated by immunohistochemistry in all the 90 matched specimens. 10 paired specimens were evaluated by quantitative real-time polymerase chain reaction (RT-PCR) analysis.

RT-PCR analysis

Total RNA from human liver tissues was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1 ug of total RNA using the First-Strand Synthesis System (Fermentas). Real-time PCR procedure was carried out using a CFX96 Real-Time System (BIO-RAD). 2X SYBR green master mixture (BIO-RAD) was used in a total volume of 10 ul. The primer sequences were as follows: TARBP1 sense 5'-TGCAAC-ATTTCACCCACTCAA-3', antisense 5'-CCCGCA-GCTAAAGGAACATC-3'. GAPDH sense 5'-TGTTG-CCATCAATGACCCC-3', antisense 5'-CTCCACG-ACGTACTCAGC-3') was used as an internal control. All reactions were run in triplicate in three independent experiments.

Immunohistochemistry

Immunohistochemical (IHC) staining was performed according to the manufacturer's protocol (Zymed®, Life Technologies, Carlsbad, CA, USA). All sections were baked 1 h at 60°C and deparaffined with xylenes and rehydrated through graded ethanol series to distilled water. Then, the sections were submerged in sodium citrate buffer and heated for antigenic retrieval. The sections were blocked by the endogenous peroxidase with 0.3% H_2O_2 for 15 min at room temperature, and then incubated with normal goat serum for 30 min at room temperature to reduce the nonspecific binding. Next, the sections were incubated with rabbit polyclonal anti-TARBP1 antibody (1:100 LSBIO) overnight at 4°C. After 3 washings in sterile phosphate-buffered saline, the sections were incubated with a biotinylated anti-rabbit secondary antibody (Zymed) followed by further incubation with streptavidin-horseradish peroxidase (Zymed) at 37°C for 30 min. Diaminobenzidine (DAB) was used for color reaction, and the anti-
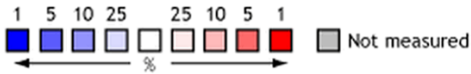
Figure 1. In Oncomine database, TARBP1 expression is up-regulated in human liver carcinomas. Oncomine heat map of TARBP1 gene expression in clinical liver carcinoma samples compared with the normal liver tissues.
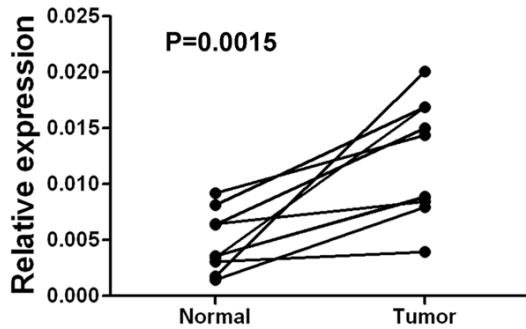

Figure 2. Expression levels of TARBP1 mRNA in liver cancer tissues. Expression levels of TARBP1 mRNA in ten paired liver cancer tissues by real-time PCR. Normal, para carcinoma (normal) liver tissues. Tumor, liver carcinoma tissues.

body was replaced by normal goat serum for negative controls. Tissue section immunoreactivities were viewed and scored separately by two independent pathologists, who were blind to the histopathological features and patient information of the samples. Scores given by the two pathologists were averaged for further comparative evaluation of TARBP1 expression. Immunoreactivities were scored by the intensity of staining (0, no staining; 1, weak = light yellow; 2, moderate = yellow brown; 3, strong = brown) and the percentage of stained cells (0, no staining; 1, 1-10%; 2, 11-35%; 3, 36-70%; 4, >70%). By multiplication of both values, a final score ranging between 0 and 12 was obtained. TARBP1 overexpression was defined as final score more than zero.

Statistical analysis

TARBP1 mRNA transcriptional levels in the tumor and matched non-tumor tissue were compared by paired sample t test. TARBP1-positive patients and TARBP1-negative patients were compared using the chi-square tests for categorical variables, and the Student t test for continuous data. Overall survival (OS) was defined from the date of operation to the date of death. Kaplan-Meier method was used to analyze overall survival (OS) and comparisons were analyzed by log-rank test. Cox's proportional hazards model was used for multivariate analysis, adjusted hazard ratios (HRs) and their 95% confidence intervals (CIs) were calculated. All the statistical analyses were evaluated using the Statistical Software Package for the Social Sciences (SPSS Inc. Chicago, IL, USA). All statistical tests were two-sided and a P value of less than 0.05 was considered statistically significant.

Results

TARBP1 is up-regulated in HCC patients

To determine whether the *TARBP1* expression levels were differentially between liver carcinoma and normal liver samples, we first queried the Oncomine database, in the meta-analysis, *TARBP1* expression significantly higher than the corresponding normal tissues in liver carcinoma with a median rank of 98.0 and a *P*-value of 6.55E-19 (**Figure 1**). To confirm this result, we obtained 10 paired liver carcinoma samples for real-time RT-PCR analysis. The result as shown in **Figure 2**, the expression level of *TARBP1* mRNA is significantly higher in tumor tissues. Furthermore, these results were confirmed by immunohistochemistry. TARBP1 positive rate was 61.1% (55/90) in tumor tissue. In non-cancerous adjacent tissues and normal tissues, TARBP1 protein was not staining. TARBP1 was predominantly present in the cytoplasm of tumor cells (**Figure 3**). These data showed that TARBP1 was significantly elevated in HCC tumor tissue.
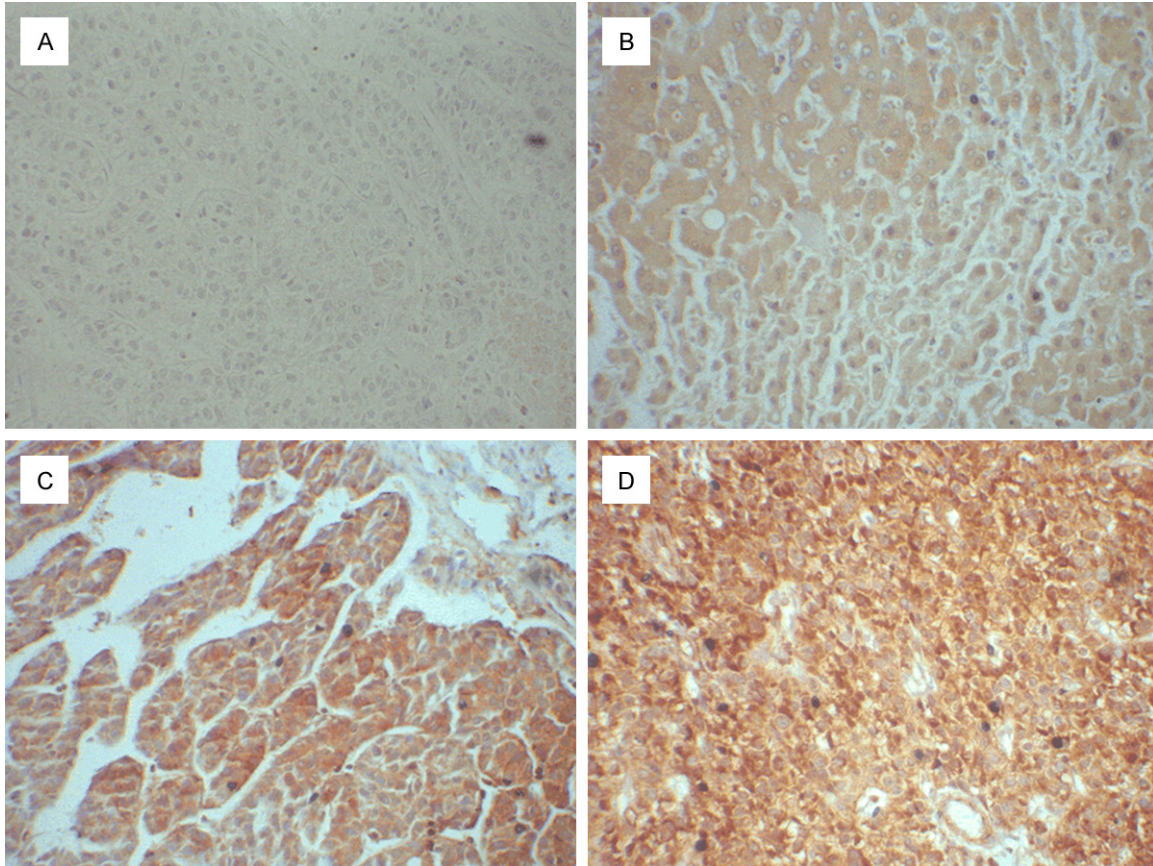
Figure 3. The expression of TARBP1 protein by immunohistochemistry. TARBP1 expression was mainly localized in the cytoplasm of tumor cells. In normal epithelial cells, TARBP1 is not expressed, in HCC cells, TARBP1 was over-expression. A. Staining of TARBP1 in normal liver tissues; B-D. Low and high expression of TARBP1 in HCC tissues.

TARBP1 correlates with clinicopathological features of HCC

For better understanding of the potential roles of TARBP1 in HCC development and progression, patients were grouped according to TARBP1 expression. TARBP1 expression was significantly associated with AJCC tumor stage (P=0.004) and clinical stage (P=0.005). However, there was no correlation of TARBP1 expression with other clinical features, such as age, gender, and tumor size (**Table 1**).

Expression of TARBP1 in HCC patients correlates with worse overall survival

After a median follow-up of 29 months (range 1-80 months), 52 (57.8%) patients died. The estimated 3-year overall survival (OS) was 42.10% (95% CI 35.22-48.98). In univariate analysis, TARBP1 expression was significantly associated with worse OS, patients with nega-

tive TARBP1 expression had a 3-year OS rate of 55.90% (95% CI 45.85-65.93), whereas those with positive TARBP1 expression had a 3-year OS rate of 33.02% (95% CI 24.65-41.38; P=0.002; **Figure 4**). In multivariate analysis (**Table 2**), factors associated with worse OS were stage III tumor (HR=3.812; 95% CI (1.579-9.423); P=0.004), and TARBP1 expression (HR=2.773, 95% CI 1.542-4.985; P=0.019). These results indicated that TARBP1 expression was an independent prognostic marker for OS of HCC patients.

Discussion

Hepatocellular carcinoma (HCC) is a disease with a high incidence and mortality rate worldwide. HCC is the most common primary liver malignancy in adults [16], in China, 360,000 incident cases and 350,000 deaths secondary to HCC occur annually [17]. HCC is a late complication of chronic liver disease, the main risk
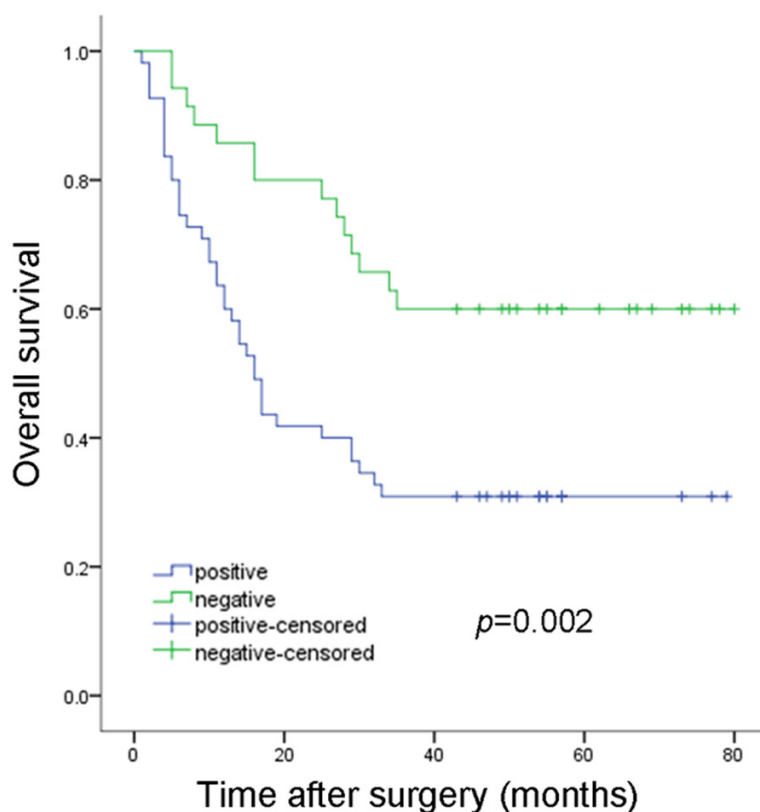
Figure 4. 3-year overall survival according to TARBP1 expression.

markers of recurrence and survival.

Human transactivation responses (TAR) RNA binding protein was initially identified as protein that bind the HIV type 1 (HIV-1) TAR RNA and activate long terminal repeat (LTR) expression in the absence and in the presence of the viral transactivator Tat [22]. It contains two double-stranded RNA binding domains (RBD). TARBP1 is a multifaceted protein with two well-characterized functions [23]. Firstly, it is an integral component of Dicer-containing complexes, and secondary, it assists Dicer in generating small RNA-induced silencing complex (RISC) [24, 25]. Consistent with the notion of miRNA-mediated restriction of viral infection in mammalian cells, loss of TARBP1 activity through its sequestration by TAR RNA resulted in the enhanced replication of HIV-1 in human cells [26]. TARBP1 has a physiological role in spermatogenesis and growth control during development. They also bind the interferon-induced dsRNA-activated protein kinase PKR, with the PKR activator PACT, and with the tumor suppressor [14].

Based on previous studies, we investigated the expression of TARBP1 in HCC and its prognostic values in predicting tumor recurrence following curative resection in the present study. Our results clearly showed that TARBP1 is up-regulated at mRNA level in ten HCC tissues compared with paired normal tissues. In addition, IHC analysis showed that TARBP1 was overexpressed in 55 of 90 (61.1%) HCC specimens. Similar observations were also made in other diseases, such as HIV [14] and epithelial skin cancer [27]. The difference in the expression of TARBP1 between tumor and non-tumor tissues indicates that TARBP1 may be a key regulator in tumorigenesis.

In this study, TARBP1 was up regulated in HCC, and it was associated with AJCC tumor stage

factors for the development of HCC include infection with hepatitis B virus (HBV) or hepatitis C virus (HCV). Hepatitis infection is believed to be the main etiologic factor in >80% of cases [18]. An overall 10 years study in Singapore show, the overall incidence of HCC in cirrhotics was 29.7% (3.0%/year) [19], Other risk factors though the management of HCC has significantly changed due to improved diagnostic technologies, development of evidence-based staging systems, and the availability of effective treatment during the past few decades, the outcome after curative resection for HCC is still poor, more than 50% of patients develop disease recurrence within 3 years after curative surgery [20]. Now there are many new strategies and target therapies in HCC, Saber Ismail show NF-κB and PRDX3 may serve as early and sensitive biomarkers for early detection of HCC and nuclear factor kappa B (NF-κB) as a target for treatment of liver fibrosis and HCC [21]. However, the mechanisms underlying its pathogenesis are still elusive, and there is still lack of marker for predicting the recurrence of HCC after resection. It is necessary to find useful

Table 2. Univariate and Multivariate analysis of the effect of covariates on overall survival for all patients

Factor	Univariate		Multivariate	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Sex				
Male	Reference			
Female	0.382 (0.229-1.761)	0.634	—	—
Age				
≥60	Reference			
<60	1.658 (0.831-3.309)	0.151	—	—
Tumor size (cm)				
<5	Reference			
≥5	0.914 (0.523-1.596)	0.751	—	—
Hepatitis				
Negative	Reference			
HBV/HCV	2.053 (1.090-8.553)	0.054	—	—
Live cirrhosis				
Absence	Reference			
Presence	0.777 (0.431-1.402)	0.403	—	—
TARBP1 expression				
Negative	Reference		Reference	
Positive	0.402 (0.217-0.744)	0.004	2.773 (1.542-4.985)	0.0019
AJCC tumor stage				
I	Reference		Reference	
II	0.150 (0.036-0.631)	0.010	—	
III	0.393 (0.201-0.768)	0.006	3.812 (1.579-9.423)	0.004
Clinical stage				
1	Reference			
2	0.301 (0.071-1.282)	0.104		
3	0.787 (0.450-1.376)	0.401	—	—

HBV, hepatitis B virus; HCV, hepatitis C virus; AJCC, American Joint Committee on Cancer.

(P=0.004) and clinical stage (P=0.005). The advance tumor stage considered as a sign of tumor progression and increased malignant potential. Our study clearly indicated that TARBP1 expression in HCC tumors was significantly associated with advanced tumor stage. In this study, we found that TARBP1 over-expression was associated with worse outcome, and it was an independent prognosis factor for HCC patients after curative resection (HR=2.773, 95% CI 1.542-4.985; *P*=0.019). These results suggest TARBP1 could be a new biomarker for HCC and a potential therapeutic target. However, the molecular mechanism connecting worse outcome and TARBP1 over-expression is unclear and remains to be elucidated.

In conclusion, HCC is the most common primary malignancy of the liver and the major cause of mortality. TARBP1, over-expression in HCC, was correlated with AJCC tumor stage and clinical stage. TARBP1 is associated with worse prognosis in HCC and could be a potential prognostic biomarker for HCC. Further studies are warranted to reveal the molecular mechanism of TARBP1 in the development and progression of HCC. To our knowledge, this is the first report to identify TARBP1 as an independent prognostic factor for HCC patients.

Disclosure of conflict of interest

None.

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