Downregulation of tumor protein 53-inducible nuclear protein 1 expression in hepatocellular carcinoma correlates with poor prognosis

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Abstract. The expression of tumor protein 53-inducible nuclear protein 1 (TP53INP1) is upregulated in certain cancers and downregulated in others. However, its expression in hepatocellular carcinoma (HCC) is not clear. The present study aimed to investigate the expression and prognostic value of TP53INP1 and its association with clinicopathological parameters in HCC. TP53INP1 expression in HCC tissue samples was examined via immunohistochemistry, western blotting and reverse transcription-quantitative polymerase chain reaction. Expression was categorized as high or low. The correlations of TP53INP1 expression with clinical characteristics and patients' prognoses were determined. TP53INP1 was frequently decreased in HCC tissues compared with adjacent non-tumorous liver tissues. This decreased expression was significantly associated with American Joint Committee on Cancer stage (P=0.014) and vascular invasion (P=0.024). Kaplan-Meier analysis further revealed that recurrence-free survival (RFS) (P=0.001) and overall survival (OS) (P=0.002) were significantly worse among patients with low TP53INP1 expression than among those with high TP53INP1 expression. In addition, multivariate analyses revealed that TP53INP1 was an independent predictor of OS [hazard ratio (HR)=2.680, 95% confidence interval (CI)=1.087-6.608, P=0.032) and RFS (HR=2.284, 95% CI=1.157-4.511, P=0.017). In conclusion, the expression of TP53INP1 was decreased in HCC, and TP53INP1 downregulation was an independent predictor of poor prognosis in patients with HCC.

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Introduction

Tumor protein 53-inducible nuclear protein 1 (TP53INP1) is an apoptotic protein involved in cell stress responses (1). It increases in abundance in response to inflammatory stress and stress-inducing agents such as heat shock, ultraviolet rays, ethanol and mutagens (2-4). TP53INP1 was first identified in a screen of stress-activated pancreatic genes in mice with acute pancreatitis (4). TP53INP1 localizes to human chromosome 8q22 (5) and encodes two nuclear isoforms, TP53INP1 and TP53INP1β. Both isoforms are related to homeodomain-interacting protein kinase-2, and regulate p53-mediated transcriptional activation of the p53-inducible gene 3, BCL2-associated X protein and p21 promoters (6).

TP53INP1 expression is downregulated in numerous human cancers, including esophageal carcinomas (7), poorly differentiated stomach adenocarcinomas (8), primary breast carcinomas (9) and pancreatic ductal adenocarcinomas (10). TP53INP1 messenger RNA (mRNA) levels were reduced in 35-59% of melanoma cell lines compared with melanocytes (11). TP53INP1 expression is enhanced in certain cancers. Ito *et al* (12) detected elevated TP53INP1 expression in anaplastic thyroid carcinomas, and Giusiano *et al* (13) reported increased TP53INP1 expression in prostate cancers. The reason why TP53INP1 is upregulated in certain cancers and downregulated in others is not clear. In addition, its expression and prognostic value in hepatocellular carcinoma (HCC) have not been reported to date.

HCC is one of the most common cancers in the world (14). It is the third major cause of cancer-associated mortalities (15) and accounts for 75-90% of all malignant tumors in adult livers (16). The aim of the present study was to analyze the expression patterns of TP53INP1 in a large series of human HCCs in order to i) identify the possible variations of TP53INP1 expression; ii) investigate its correlation with clinicopathological parameters; and iii) evaluate its prognostic value.

Materials and methods

Patient management and tissue samples. The present study was performed in accordance with the reporting recommendations for tumor marker prognostic studies guidelines (17). The

institutional ethics committee of Southern Hospital (Guangzhou, China) approved the protocol, and all enrolled subjects provided written informed consent.

Fresh HCC tissue samples and matched adjacent non-tumorous tissues were collected from 65 HCC patients who underwent resection at the Digestive Disease Research Institute of Southern Hospital between March 2008 and March 2011. The enrolled patients i) had a conclusive pathologic diagnosis of HCC; ii) had received curative resection, which was defined as macroscopically complete removal of the tumor; and iii) had available detailed clinicopathological data. Patients were excluded if they had received adjuvant chemotherapy or radiotherapy prior to surgery, or if there was evidence of other malignancies. The detailed clinicopathological characteristics of the HCC patients included in the current study are presented in Table I.

Patients were followed up until August 31, 2014. Among the 65 patients, 9 (13.8%) were lost to follow-up. Tumor recurrence confirmation was based on typical appearances on magnetic resonance imaging and/or computed tomography scans, as well as elevated α-fetoprotein protein (AFP) levels. The median follow-up period was 31 months (range, 1-71 months). Tumor differentiation was based on the criteria proposed by Edmondson and Steiner (18). Tumor stage was defined according to the American Joint Committee on Cancer (AJCC)/International Union against Cancer tumor node metastasis classification system (19).

Immunohistochemistry assay. Immunostaining was performed on 4-μm sections of paraffin-embedded tissue specimens. The sections were deparaffinized with xylene and rehydrated in a graded alcohol series. Antigen retrieval was carried out in a microwave oven in a sodium citrate solution (pH 8.0). Endogenous peroxidase was inactivated by incubating the samples in 3% H_2O_2 at room temperature for 20 min. Upon blocking with goat serum (Wuhan Boster Biological Engineering Co., Ltd.) at room temperature for 30 min, the samples were incubated with rabbit polyclonal anti-TP53INP1 antibody (catalog no. AP11890b; 1:50; Abgent, Inc., San Diego, CA, USA) at 4˚C overnight in a moist chamber. They were then washed thoroughly with PBS and incubated with secondary antibodies (catalog no. HSP0007; 1:200; Shanghai Mjol Biological Technology Co., Ltd.) at 37˚C for 30 min, conjugated to peroxidase (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). Staining (which was brown-colored) was visualized using a 3,3'-diaminobenzidine kit (Zhongshan Golden Bridge Biotechnology Co., Ltd.). Upon counterstaining with hematoxylin, the samples were dehydrated in a graded alcohol series and mounted. Negative controls were prepared in the absence of primary antibody.

Immunohistochemical staining was evaluated by two independent observers who were blinded to the clinical data. Concordance was achieved in 94% of the cases, and disagreements were resolved by consensus (20). Each sample was scored according to the intensity of the staining (no staining=0, weak staining=1, moderate staining=2 and strong staining=3) and the percentage of stained cells (<5%=0, 5-25%=1, 26-50%=2, 51-75%=3 and 76-100%=4). The percentage of cells at each intensity was multiplied by the corresponding intensity value to obtain an immunostaining score ranging from 0 to 12. The scores were combined to obtain an overall mean score. Using this assessment system, the optimal cutoff values were as follows: 0-3 (low) and 4-12 (high).

Table I. Clinicopathological features of 65 patients with hepatocellular carcinoma.

Variables	Value
Median age (range), years	49.9 (18-83)
Gender, n	
Male	59
Female	6
HBsAg expression, n	
Positive	56
Negative	9
AFP levels, n	
>400 ng/ml	30
≤400 ng/ml	35
Liver cirrhosis, n	
Absent	40
Present	25
Vascular invasion, n	
Absent	11
Present	54
Intrahepatic metastasis, n	
Absent	53
Present	12
Tumor size, n	
≤5 cm	23
>5 cm	42
Tumor number, n	
Single	55
Multiple	10
Tumor differentiation, n	
Well	23
Moderate	31
Poor	11
AJCC stage, n	
I/II	56
III/IV	9

HBsAg, hepatitis B surface antigen; AFP, α-fetoprotein; AJCC, American Joint Committee on Cancer.

Western blot analysis. Proteins were extracted in radioimmunoprecipitation buffer (EMD Millipore, Billerica, MA, USA). Protein concentration was determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Samples with equal amounts of total protein were separated on 12% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Upon blocking in TBS/Tween-20 containing 5% non-fat milk powder at room temperature for 5 min with agitation, the membranes were incubated for 1 h with anti-TP53INP1 rabbit

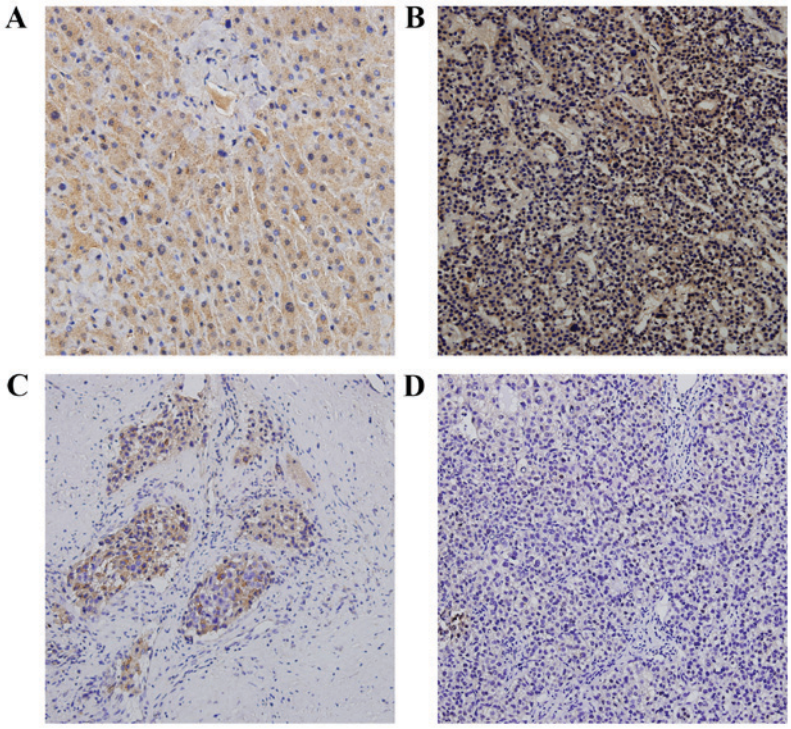

Figure 1. Immunohistochemical analysis of TP53INP1 expression in HCC. (A) TP53INP1 was strongly expressed in adjacent non-tumorous liver tissues (magnification, x200). TP53INP1 expression decreased in (B) well (magnification, x200), (C) moderately (magnification, x200) and (D) poorly differentiated HCC tissues (magnification, x200).

polyclonal antibody (catalog no. AP11890b; 1:250; Abgent, Inc.) and anti-β-actin mouse monoclonal antibody (catalog no. CW0096; 1:1,000; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) at 4˚C overnight. Upon incubation of the membranes with secondary antibodies (catalog no. HSP0007; 1:200; Shanghai Mjol Biological Technology Co., Ltd.) at room temperature for 3 h, immunoreactive bands were visualized by enhanced chemilumiscence using a GeneGnome HR Bioimaging System (Syngene, Frederick, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from tissues using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), and complementary DNA libraries were generated from total RNA using a High-Capacity cDNA Archive kit according to the manufacturer's protocol (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-qPCR was performed in triplicate using the SYBR-Green system on a LightCycler 480 Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany). Relative mRNA levels were calculated according to the quantification cycle (Cq) values corrected for GAPDH expression using the $2^{-\Delta\Delta Cq}$ method as follows: ΔΔCq=ΔCq (treatment)-ΔCq (control) or ΔCq=Cq (target genes)-Cq (GAPDH). The primer sequences were as follows: TP53INP1, 5'-GCACCCTTCAGT CTTTTCCTGTT-3' (forward) and 5'-GAGAAAGCAGGA ATCACTTGTATC-3' (reverse); and GAPDH, 5'-GAAGGT GAAGGTCGGAGT-3' (forward) and 5'-GAAGATGGTGAT GGGATTTC-3' (reverse).

Statistical analysis. All statistical analyses were carried out using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA). Differences between two independent groups were analyzed using the Student's *t*-test. The clinicopathological features of HCC were analyzed using the Pearson's χ^2 test. Recurrence-free survival (RFS) and overall survival (OS) were calculated using the Kaplan-Meier method, and significance was assessed using the log-rank test. RFS was defined as the interval between the date of surgery and the date of detection of a recurrent tumor. OS was defined as the interval between the date of surgery and the date of mortality or last follow-up. Independent prognostic factors for OS and RFS were identified using the Cox proportional hazards regression model. Data are presented as the mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TP53INP1 expression is downregulated in HCC tissues. TP53INP1 expression was significantly decreased in HCC tissues compared with adjacent non-tumorous tissues, as determined via western blotting and immunohistochemistry. TP53INP1 was predominantly localized in the cytoplasm of hepatic cells, with little staining was present in the nuclei, as visualized via immunohistochemistry. Heavy TP53INP1 staining was observed in the epithelial cells in normal-appearing mucosa adjacent to HCC cells, whereas TP53INP1 staining in HCC cells was faint or absent (Fig. 1). In western blot analyses, TP53INP1 expression was lower in 8 of 9 HCC tissue samples than in matched adjacent non-tumorous tissue (Fig. 2).

To further examine TP53INP1 expression, several sample sets were analyzed via RT-qPCR. Notably, TP53INP1 mRNA expression was significantly lower in HCC tissues (0.4103±0.03674) than in adjacent non-tumorous tissues (0.6851±0.05825, P=0.0001) (Fig. 3).

Table II. Association of TP53INP1 messenger RNA expression with clinicopathological characteristics of 65 patients with hepatocellular carcinoma.

Variables	Patients, n	TP53INP1 expression		P-value
		Low (n=32)	High (n=33)	
Age, years				0.897
<50	34	17	17	
≥50	31	15	16	
Gender				0.968
Male	59	29	30	
Female	6	3	3	
HBsAg expression				0.304
Positive	56	29	27	
Negative	9	3	6	
AFP levels, ng/ml				0.702
>400	30	16	14	
≤400	35	18	17	
Liver cirrhosis				0.875
Absent	25	12	13	
Present	40	20	20	
Vascular invasion				0.024
Absent	11	2	9	
Present	54	30	24	
Intrahepatic metastasis				0.223
Absent	53	28	25	
Present	12	8	4	
Tumor size, cm				0.056
≤5	23	15	8	
>5	42	17	25	
Tumor number				0.110
Single	54	29	25	
Multiple	42	17	25	
Tumor differentiation				0.644
Well	23	12	11	
Moderate	31	16	15	
Poor	11	4	7	
AJCC stage				0.014
I/II	56	31	25	
III/IV	9	1	8	

HBsAg, hepatitis B surface antigen; AFP, α-fetoprotein; AJCC, American Joint Committee on Cancer; TP53INP1, tumor protein 53-induced nuclear protein 1.

Expression of TP53INP1 and its clinicopathological relevance in hepatic tissues. To investigate the significance of TP53INP expression in HCC, the association between TP53INP mRNA levels and the clinicopathological characteristics of 65 HCC patients were evaluated in the present study. TP53INP1 mRNA expression was categorized as high or low. As shown in Table II, low expression of TP53INP1 mRNA closely correlated with AJCC stage (P=0.014) and vascular invasion (P=0.024). There were no significant differences in other clinical characteristics between the high and low expression groups.

Low expression of TP53INP1 predicts poor prognosis in HCC patients. The potential association between TP53INP1 expression and survival (RFS and OS) was retrospectively evaluated via Kaplan-Meier analysis. RFS (Fig. 4A) and OS (Fig. 4B) were significantly worse in HCC patients expressing low TP53INP1 levels compared with those expressing high TP53INP1 levels,

Table III. Univariate analysis of factors associated with RFS and OS in patients with hepatocellular carcinoma.

	RFS		OS	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years (<50 vs. ≥50)	1.111 (0.600-2.059)	0.737	0.667 (0.310-1.438)	0.302
Gender (male vs. female)	1.200 (0.468-3.075)	0.704	1.223 (0.368-4.067)	0.743
HBsAg expression (negative vs. positive)	1.420 (0.555-3.632)	0.464	1.656 (0.498-5.505)	0.411
AFP levels, ng/ml (≤400 vs. >400)	1.816 (0.972-3.391)	0.061	2.812 (1.281-6.169)	0.010
Liver cirrhosis (absent vs. present)	0.902 (0.478-1.703)	0.751	1.118 (0.512-2.442)	0.780
Vascular invasion (absent vs. present)	3.004 (1.416-6.373)	0.004	4.275 (1.848-9.889)	0.001
Intrahepatic metastasis (absent vs. present)	1.833 (0.895-3.753)	0.098	1.759 (0.709-4.363)	0.223
Tumor size, cm (≤5 vs. >5)	1.447 (0.737-2.841)	0.283	1.377 (0.602-3.150)	0.448
Tumor number (single vs. multiple)	1.729 (0.821-3.641)	0.150	1.744 (0.702-4.328)	0.231
Differentiation (poor/moderate vs. well)	0.521 (0.338-0.801)	0.003	0.432 (0.251-0.744)	0.002
AJCC stage (I/II vs. III/IV)	2.224 (1.016-4.869)	0.045	2.943 (1.182-7.327)	0.020
TP53INP1 expression (low vs. high)	2.604 (1.345-5.039)	0.005	3.403 (1.436-8.063)	0.005

RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; AFP, α-fetoprotein; AJCC, American Joint Committee on Cancer; TP53INP1, tumor protein 53-induced nuclear protein 1.

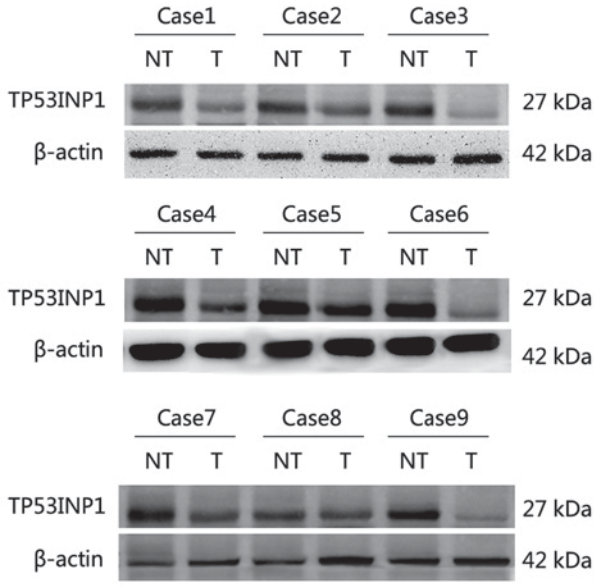

Figure 2. TP53INP1 protein expression was detected in nine paired hepatocellular carcinoma tissues and matched adjacent non-tumorous tissues by western blotting. T, tumorous; NT, non-tumorous; TP53INP1, tumor protein 53-induced nuclear protein 1.

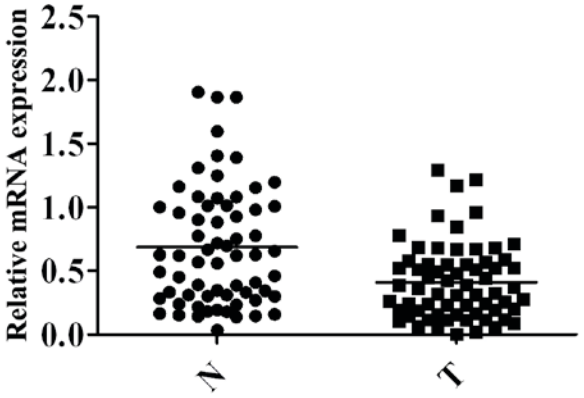

Figure 3. Relative messenger RNA expression of tumor protein 53-induced nuclear protein 1 in hepatocellular carcinoma tissues was decreased compared with that in matched adjacent non-tumorous tissues, as determined by reverse transcription-quantitative polymerase chain reaction (n=65, P<0.001). T, tumor; N, normal.

with median survival times of 10 and 38 months, respectively (P=0.003).

Twelve clinicopathological variables were included in a univariate analysis, including age, gender, serum hepatitis B surface antigen (HBsAg) levels, serum AFP levels, liver cirrhosis, vessel invasion, intrahepatic metastasis, tumor size, tumor number, tumor differentiation, AJCC stage and TP53INP1 expression. Of these, tumor differentiation, AJCC stage, vascular invasion and TP53INP1 expression were significant prognostic factors of RFS and OS in univariate analysis (Table III). In addition, multivariate analysis (Table IV) revealed that TP53INP1 was also an independent prognostic factor for both RFS [hazard ratio (HR)=2.284, 95% confidence interval (CI)=1.157-4.511, P=0.017] and OS (HR=2.680, 95% CI=1.087-6.608, P=0.032) (Table III). Thus, low expression of TP53INP1 may serve as a prognostic indicator for patients with HCC.

Discussion

TP53INP1 is a stress-induced protein that serves a role in p53-mediated apoptosis and cell cycle arrest (2,4,21). Its expression is downregulated in stomach (8), pancreatic (10) and inflammation-mediated colic carcinomas (22), but upregulated in medullary thyroid carcinomas (23) and prostate cancers (24). Therefore, it is possible that TP53INP1 can act either as a tumor suppressor or an oncoprotein depending on the tumor microenvironment or the tissue type. To date,

Table IV. Multivariate analysis of factors associated with RFS and OS in patients with hepatocellular carcinoma.

Variables	RFS		OS	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years (<50 vs. ≥50)	2.063 (0.971-4.387)	0.060	1.227 (0.499-3.017)	0.656
Gender (male vs. female)	1.500 (0.468-4.802)	0.495	1.635 (0.400-6.674)	0.493
HBsAg expression (negative vs. positive)	1.833 (0.561-5.994)	0.316	1.565 (0.375-6.537)	0.539
AFP levels, ng/ml (≤400 vs. >400)	1.587 (0.838-3.004)	0.156	2.512 (1.135-5.560)	0.023
Liver cirrhosis (absent vs. present)	1.060 (0.419-2.681)	0.902	1.398 (0.460-4.248)	0.554
Vascular invasion (absent vs. present)	2.310 (1.065-5.012)	0.034	2.841 (1.172-6.884)	0.021
Intrahepatic metastasis (absent vs. present)	2.043 (0.558-7.482)	0.281	1.842 (0.379-8.944)	0.449
Tumor size, cm (≤5 vs. >5)	2.214 (0.839-5.839)	0.108	1.889 (0.589-6.064)	0.285
Tumor number (single vs. multiple)	1.354 (0.462-3.967)	0.580	1.350 (0.388-4.697)	0.637
Differentiation (poor/moderate vs. well)	1.584 (0.575-4.236)	0.672	1.736 (0.458-5.872)	0.413
AJCC stage (I/II vs. III/IV)	0.695 (0.220-2.199)	0.536	0.867 (0.219-3.438)	0.839
TP53INP1 expression (low vs. high)	2.284 (1.157-4.511)	0.017	2.680 (1.087-6.608)	0.032

RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; AFP, α-fetoprotein; AJCC, American Joint Committee on Cancer; TP53INP1, tumor protein 53-induced nuclear protein 1.

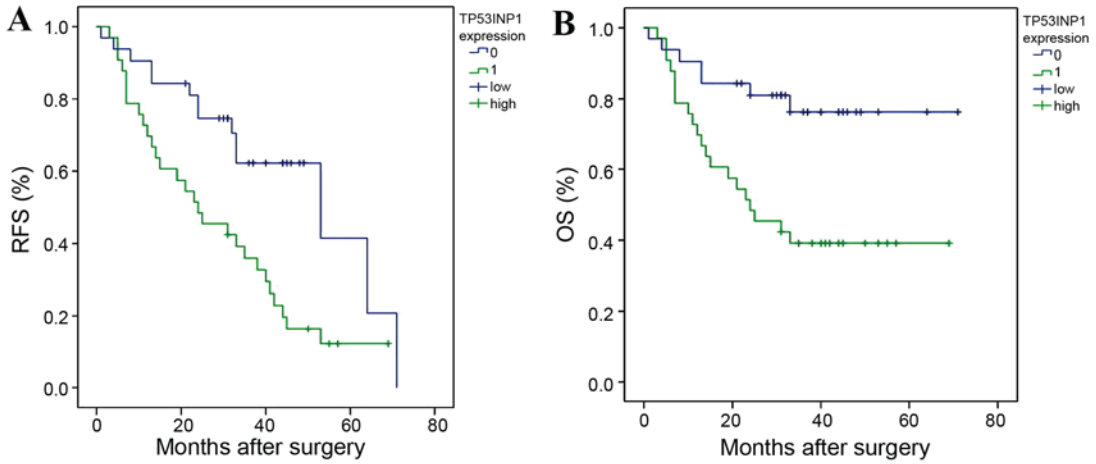

Figure 4. Kaplan-Meier survival curves for (A) RFS and (B) OS in patients with hepatocellular carcinoma, according to their TP53INP1 expression levels. Blue, patients with low TP53INP1 expression; green, patients with high TP53INP1 expression; TP53INP1, tumor protein 53-induced nuclear protein 1.

TP53INP1 expression and its prognostic value in HCC have been unclear.

The present study systematically examined the expression of TP53INP1 in HCC tissue samples. TP53INP1 protein levels were measured via western blotting and immunohistochemistry, and TP53INP1 mRNA levels were quantitated via RT-qPCR. All analyses revealed significantly lower expression of TP53INP1 in HCC tissue samples than in paired samples of non-tumorous adjacent regions. To the best of our knowledge, the present study is the first to report TP53INP1 downregulation in HCC.

TP53INP1 expression is high in HCC adjacent non-tumorous tissues and low in HCC tissue, suggesting that loss of TP53INP1 maybe contribute to HCC progression. Seux *et al* (25) observed that TP53INP1 silencing increased the migration of mouse embryonic fibroblasts and pancreatic cancer cells. Seillier *et al* (26) demonstrated that TP53INP1 interacted with autophagy-related 8 proteins to induce autophagy-dependent cell death in U2OS cells. Future studies should focus on clarifying the mechanisms of TP53INP1 expression in HCC.

Jiang *et al* (8) reported that reductions in TP53INP1 expression in gastric adenocarcinomas were associated with poor prognosis. Conversely, Giusiano *et al* (24) observed that TP53INP1 overexpression was an unfavorable prognostic factor in prostate cancer. The present study is the first to reveal a positive impact of TP53INP1 expression on survival in HCC. In addition to confirming that the expression of TP53INP1 was downregulated in HCC tissues, the present results further revealed that low TP53INP1 expression significantly correlated with advanced AJCC stage and vascular invasion, and that decreased expression of TP53INP1 predicted poor prognosis in patients with HCC following hepatectomy. Lastly, TP53INP1 expression was a prognostic indicator of RFS and OS, independently of other clinicopathological variables, in a multivariate analysis.

In conclusion, the present study identified for the first time the downregulation of TP53INP1 in human HCC tissues, which was closely associated with AJCC stage and vascular invasion in patients with HCC. Our findings also suggest that low expression of TP53INP1 may serve as a potent prognostic marker for patients with HCC.

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