

Correlation Between Poor Prognosis and Lower *TPPP* Gene Expression in Hepatocellular Carcinoma

YOSHIKUNI INOKAWA^{1,2}, FUMINORI SONOHARA^{1,2}, MITSURO KANDA¹, MASAMICHI HAYASHI¹,
YOKO NISHIKAWA¹, HIROYUKI SUGIMOTO¹, YASUHIRO KODERA¹ and SHUJI NOMOTO^{1,2}

¹Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Nagoya, Japan;

²Department of Surgery, Aichi Gakuin University School of Dentistry, Nagoya, Japan

Abstract. *Background/Aim:* Post-resection recurrence of hepatocellular carcinoma (HCC) tends to derive from multicentric origins, which indicates that the background liver microenvironment affects carcinogenesis. *Materials and Methods:* We obtained control liver samples [super normal (SN)] from 11 patients with secondary metastatic liver malignancies and used expression and methylation arrays to compare them with non-cancerous liver tissue from a patient with typical HCC with chronic hepatitis C (corresponding normal (CN)). *Results:* The expression array showed that gene expression of tubulin polymerization-promoting protein (TPPP) was lower in CN compared with SN. The methylation array showed a greater TPPP methylation index for CN than for SN. Transcripts of TPPP differed significantly among SN (n=11), CN (n=179), and tumor tissue of HCC (n=179) (median of 116, 4.60, and 2.63, respectively, $p < 0.001$). Multivariate analysis showed lower TPPP expression in tumor than in normal tissue (ratio < 0.3 , $n=57$) to independently predict poor overall survival ($p=0.031$). *Conclusion:* Significantly lower TPPP expression was found in HCC and CN tissue compared to SN and indicated poor prognosis.

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most frequent cause of cancer-related death worldwide (1), and its incidence is steadily rising. Despite therapeutic options such as hepatic resection (2), liver transplantation (3), radiofrequency ablation (4), transcatheter arterial chemoembolization (5), and sorafenib (6), the prognosis for patients with advanced HCC remains poor (7, 8). The mechanisms of hepatocarcinogenesis thus urgently need clarification (9).

Correspondence to: Shuji Nomoto, Department of Surgery, Aichi Gakuin University School of Dentistry, Nagoya 464-8651, Japan. Tel: +81 527592167, Fax: +81 527592107, e-mail: snomoto@dpc.agu.ac.jp

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Up to 70% of patients develop tumor recurrence within 5 years of resection for HCC (2), mostly in the remnant liver (10). Recurrence in remnant liver is considered to reflect intrahepatic metastasis (IM) or multicentric occurrence (MO) (11). We previously reported clonal analyses that showed recurrent HCC genotypes, mutations of the mitochondrial genome (12), or patterns of promoter hypermethylation in several tumor suppressor genes in tumor cells (13), and found MO to be a more common source of recurrence than IM. This suggests that the background liver microenvironment has a larger role in hepatocarcinogenesis than does the primary tumor.

We developed a multi-array analysis from expression and methylation arrays of background liver (14), which found tubulin polymerization-promoting protein (TPPP) to be an HCC-related gene in parenchymal tissue that surrounds HCC tumors. Although TPPP (also called p25) was found to be a brain-specific protein that binds to tubulin and microtubules (15), to our knowledge, its role in HCC has not been studied. We, therefore, investigated its expression, methylation and prognostic effect in patients who underwent liver resection for HCC.

Materials and Methods

Sample collection and DNA preparation. Control samples, termed super normal (SN) liver, were obtained from normal liver tissues of 11 patients with metastatic secondary malignancies to the liver who underwent liver resection at our Department. Their primary diseases included five with colorectal cancer, two with gastrointestinal stromal tumor, and one each with gastric cancer, esophageal cancer, cervical cancer, and tongue cancer. We compared these samples with a corresponding normal tissue specimen from a typical case of HCC that was generated from chronic hepatitis C (CN). This latter patient was a 58-year-old man with liver cirrhosis who had undergone a liver resection from which the CN tissue was obtained; his recurrence presented 3 years after resection of the primary lesion. DNA and RNA were extracted from the SN and CN tissues. In making pathological slides to extract DNA and RNA, we carefully confirmed that the corresponding normal tissue had no cancer tissues by pathological diagnoses. Expression profiling and

methylation arrays were used to compare SN and CN. We identified genes that differed in expression and rate of methylation.

We also collected primary HCC tissues and corresponding noncancerous tissues taken during liver resection at Nagoya University Hospital between 1994 and 2008 from 179 HCC patients (mean age=62.2±9.9 years; range=21-84 years; 152 men and 27 women). The median follow-up period was 67.6 months (range=0.3-208.9 months). All patients gave written informed consent, as required by the Institutional Review Board. All these tissues were diagnosed histologically as HCC. Samples were frozen immediately in liquid nitrogen and stored at -80°C until needed. Genomic DNA was taken from tissue samples by proteinase K digestion, followed by phenol/chloroform extraction.

RNA isolation and microarray procedures. Total RNA was extracted from the SN and CN samples using the Qiagen miRNeasy Mini kit (Toronto, ON, Canada). RNA quality was assessed as an RNA integrity number of ≥8 using an Agilent 2100 Bioanalyzer (Agilent, Mississauga, ON, Canada). To eliminate personal background, total RNA of 11 SN samples were mixed. Total RNA was labeled with cyanine-3 dye using a Quick Amp Labeling Kit (Agilent) and hybridized to Agilent Whole Human Genome (4×44 K) microarrays for 17 h in a rotating SciGene model 700 oven (Sunnyvale, CA, USA). Arrays were scanned by a DNA Microarray Scanner (Agilent). Data were extracted using Feature Extraction Software 10.5.1.1 (Agilent) and statistically analyzed by default settings on GeneSpring GX 11.0.1 software (Agilent) (16).

Methylation array platform. The Illumina Infinium Human Methylation 27 Bead Chip protocol uses 500 ng to 1 µg of bisulfite-converted DNA (17). Methylation array analysis was performed as described previously (18, 19).

Methylation-specific PCR (MSP). DNA from HCC cell lines, 179 of resected HCC tissues (HTs) and adjacent normal tissues (NTs) were subject to bisulfite treatment. Briefly, 2 µg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard purification resin (Promega Corp., Madison, WI, USA), treated with NaOH, precipitated with ethanol and resuspended in water. Primer pairs were used to detect methylation (sense, 5'-GGT AGA GGT TTC GGT GCG TT-3'; antisense, 5'-ACG AAA CTC TCC AAC CGC CG-3'; 198-bp product) and non-methylation (sense, 5'-GTG GGG GTG GGG TTG TTA AT-3'; antisense, 5'-AAC ATC CAA CAC CCA CCC AA-3'; 59-bp product) of the *TPPP* promoter region near exon 1. MSP amplification was performed as follows: denaturation at 94°C for 3 min followed by 38 cycles at 94°C for 10 s, 60°C for 8 s and 72°C for 6 s. Unmethylated-specific PCR (UNMSP) amplification was performed as follows: denaturation at 94°C for 3 min followed by 42 cycles at 94°C for 8 s, 62°C for 5 s and 72°C for 3 s. PCR products were loaded directly onto 3% agarose gels, stained with ethidium bromide and visualized under UV light.

Reverse transcription-polymerase chain reaction (RT-PCR). *TPPP* mRNA expression was analyzed by semi-quantitative RT-PCR and real-time RT-PCR. Total RNA (10 µg) isolated from nine HCC cell lines, primary HTs and NTs were used to generate cDNAs, which were then amplified by PCR using primers for *TPPP* (sense, 5'-AGG ACT GCC AGG TGA TCG AC-3' in exon 3; antisense 5'-CTT GCC CTC GAT GAG CCT GT-3' in exon 4), which amplified a 200 bp

product. PCR was performed using the following parameters: initial denaturation at 94°C for 3 min, followed by 36 cycles of 94°C for 10 s, 60°C for 8 s and 72°C for 6 s. RT-PCR of β-actin was performed to confirm equal amounts of cDNA template in each amplification. Each PCR product was loaded directly onto 3% agarose gels, stained with ethidium bromide and visualized under UV light.

5-Aza-2'-deoxycytidine (5-aza-dC) treatment. HCC cell lines were treated with 1 µM 5-aza-dC (Sigma-Aldrich, St. Louis, MO, USA) to inhibit DNA methylation. Cells (1.5×10⁶) were cultured for 6 days with medium changes on days 1, 3 and 5. On day 6, cells were harvested, RNA was extracted and RT-PCR was performed as described above.

Real-time quantitative RT-PCR (qRT-PCR). qRT-PCR was performed using a SYBR Green PCR Core Reagents kit (Applied Biosystems, Foster City, CA, USA) under the following conditions: one cycle at 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and at 60°C for 30 s. SYBR Green emission was detected in real time using a StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The primers used for qRT-PCR were the same as those described above for RT-PCR. Samples were standardized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). qRT-PCR was performed at least three times, including negative controls without a template. *TPPP* expression was decided as the value of $TPPP \times 10^3 / GAPDH$ in each sample.

Statistical analysis. Continuous variables are expressed as medians (range). Comparisons were made using the Mann-Whitney *U*-test. Categorical variables were compared using χ^2 tests or Fisher's exact tests, where appropriate. Overall survival (OS) rates were analyzed by Kaplan-Meier and log-rank tests. All statistical analyses were performed on JMP software version 9.0.2 (SAS International Inc., Cary, NC, USA). A value of $p < 0.05$ was considered significant.

Results

Expression profiling and methylation array analysis. To identify novel tumor-related genes in background normal liver, we first looked for genes with lower expression in CN compared to SN tissue. Expression array analysis revealed that in CN, expression of *TPPP*, which is involved in neuropeptide metabolism, was strongly down-regulated (2.236-fold log₂ ratio) compared to SN (Table I).

Methylation array analysis showed continuous β values of 0.731 for CN tissue versus 0.384 for SN tissue, indicating a high degree of methylation in the CN sample from the hepatitis C virus (HCV)⁺ patient (Table II). This implies that a mechanism of *TPPP* down-regulation is through hypermethylation of its promoter region in background liver tissue.

Methylation status of HCC cell lines. We next investigated the methylation status of *TPPP* in nine HCC cell lines, using primers for methylated and unmethylated *TPPP*. For methylation-specific (MSP) analysis, bands of appropriate size were observed for HLE, HuH2, HuH7 and SK-Hep1 cell lines, whereas appropriate bands were identified in all cell

Table I. Expression array analysis of tubulin polymerization-promoting protein (TPPP) in super normal (SN) and corresponding normal (CN) liver tissues.

Probe set ID	Gene symbol	Log2 ratio	SN signal	Detection	CN signal	Detection	Chromosomal location
A_23_P117464	TPPP	-2.236	357.4	Present	192.6	Present	Chr5p15.3

lines for unmethylated *TPPP* (Figure 1a). These results indicate partial methylation of *TPPP* in HLE, HuH2, HuH7 and SK-Hep1 and no methylation in HepG2, Hep3B, HLF, HuH1 and PLC/PRF/5 cells.

Inhibitory effect of methylation on TPPP expression in HCC cell lines. To confirm that *TPPP* expression was silenced by promoter hypermethylation, we analyzed *TPPP* mRNA expression in nine HCC cell lines before and after treatment with the DNA methylation-inhibitory agent 5-aza-dC; *TPPP* expression was clearly reactivated after 5-aza-dC treatment in three cell lines (HLE, HLF and SK-Hep1), as shown by semi-quantitative RT-PCR (Figure 1b).

MSP and non-MSP of SN, normal and tumor tissues from pw HCC. We next assessed *TPPP* promoter hypermethylation in SN, normal and tumor tissues from 179 patients with HCC. Overall, 28.4% (51/179) of tumor samples displayed *TPPP* promoter hypermethylation compared with 9.4% (17/179) of normal samples (Table III). SN tissues showed no methylation in all 11 cases (Figure 2a). Thus, hypermethylation of *TPPP* was significantly more frequent in tumor tissues than CN tissues ($p < 0.001$) and SN ($p = 0.038$). Although SN and CN were not significant different ($p = 0.602$), CN tended to be more methylated than SN (Table III). Four representative cases of methylated and unmethylated status are shown in Figure 2b.

qRT-PCR of SN and CN tissue of HCC cases. Overall, *TPPP* expression (relative to *GAPDH*) was significantly lower in CN samples ($n = 179$) than in SN tissues ($n = 11$), with median *TPPP* $\times 10^3 / \text{GAPDH}$ ratios for SN of 116 (range=31.0-348) vs. CN of 4.60 (range=0.500-64.3) ($p < 0.001$), and lower still in tumor tissues (median *TPPP* $\times 10^3 / \text{GAPDH}$ ratio=2.63, range=0.0240-300) than in CN tissues ($p < 0.001$) (Figure 3). The *TPPP* expression index was calculated as the tumor tissue expression level divided by the expression in adjacent normal tissue. The methylation status evaluated by MSP and expression indices did not show any statistically significant correlation (data not shown).

Correlation between down-regulation of TPPP and HCC clinicopathological characteristics. A lower *TPPP* expression index was significantly correlated with poorly differentiated HCC ($p = 0.038$) and high alpha-feto-protein

(AFP) level ($p = 0.012$) (Table IV). The group of patients with *TPPP* expression indices < 1.0 ($n = 121$) had worse OS after surgery than the group with *TPPP* expression indices ≥ 1.0 ($n = 58$) ($p = 0.047$; Figure 4a). Moreover, the 57 patients with *TPPP* expression indices < 0.3 had significantly worse prognoses than other patients ($p < 0.001$; Figure 4b).

Univariate analysis of OS identified significant correlations with viral infection (HCV vs. other) ($p = 0.032$), liver cirrhosis ($p = 0.036$), multiple tumors ($p = 0.016$), vascular invasion ($p < 0.001$), pathological type (well-/moderately differentiated vs. poorly differentiated; $p = 0.036$), AFP level (≥ 20 ng/ml; $p = 0.001$), advanced tumor stage (Japan Stage I-II vs. III-IV; $p = 0.028$) and *TPPP* expression index < 0.3 ($p < 0.001$) (Table V).

Multivariate analysis showed OS to be significantly correlated with HCV infection ($p < 0.001$), vascular invasion ($p < 0.001$), higher AFP level ($p = 0.038$) and *TPPP* expression index < 0.3 ($p = 0.031$) (Table V).

Discussion

Usually, HCC develops within an established background of chronic liver disease (20). The high incidence of intrahepatic recurrence is a major problem in HCC therapy (10, 21). HCC has a peculiar progression pattern; unlike most other malignancies, most patients with HCC have lesions only in their livers without metastasis to other organs (22). The background liver environment in HCC, thus, merits intense investigation.

We previously reported that HCC recurrence after liver surgery is mainly MO not IM (12, 13), which indicates that recurrent lesions derive from oncogenic activities of background liver parenchyma rather than primary lesions. This implies that background liver tissue is a greater factor in HCC than is tumor tissue. We developed a multi-array analysis to investigate gene expression in these tissues (14).

Here, our multi-array analysis detected the gene for *TPPP*, identified as a brain-specific protein (also called p25) (15, 23). Only a few reports have been published regarding its effect in carcinogenesis (24-26), and to our knowledge, no studies have been published of its role in HCC or liver tissue. However, GeneCards indicates that among internal organs, *TPPP* is strongly expressed in liver tissue (27), which concurred with our array analysis and RT-PCR showing *TPPP* mRNA to be

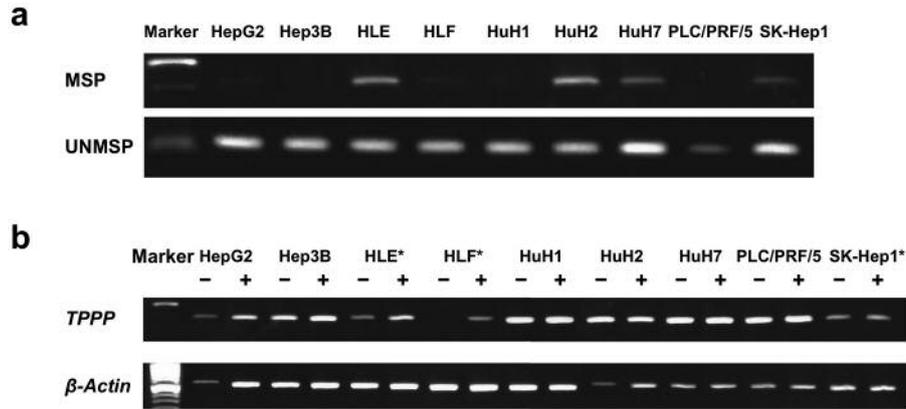


Figure 1. Results of polymerase chain reaction for nine hepatocellular carcinoma (HCC) cell lines. a: polymerase chain reaction (PCR) for methylation-specific (MSP) and unmethylated (UNMSP) tubulin polymerization-promoting protein (TPPP) gene. The HLE, HuH2, HuH7 and SK-Hep1 cell lines were MSP⁺; but all cell lines were UNMSP⁺. b: Semi-quantitative reverse transcription PCR revealed reactivation of TPPP mRNA expression in three out of nine HCC cell lines (HLE, HLF and SK-Hep1; shown with asterisks) after treatment with the DNA methylation-inhibitory agent 5-aza-2'-deoxycytidine.

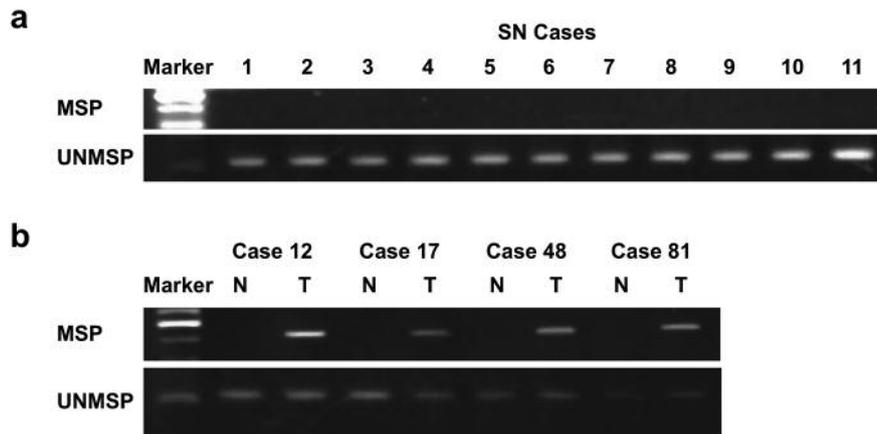


Figure 2. Results of polymerase chain reaction for methylation-specific (MSP) and unmethylated (UNMSP) tubulin polymerization-promoting protein (TPPP) gene in super normal (SN) cases (a) and hepatocellular carcinoma cases (b). a: SN tissues showed no methylation in all 11 cases. b: Representative four cases show hypermethylation of the promoter region of TPPP gene in tumor tissues, but no methylation in normal tissues.

highly expressed in SN liver parenchyma, significantly down-regulated in CN tissues and even further down-regulated in tumor tissues. We consider that decreased TPPP expression is important in hepatocarcinogenesis, and its down-regulation might reflect promoter methylation.

Schofield *et al.* investigated TPPP and showed that its overexpression reduces, and its knockdown increases, proliferation in the U2OS cell line (28). They studied the effect of TPPP on the cell cycle and found it to inhibit G₁/S-phase transition. Lehotzky *et al.* also showed that siRNA knockdown of TPPP inhibited differentiation and promoted proliferation, which implies an important role for TPPP in oligodendrogenesis (29). An immunohistochemical study showed TPPP expression in non-neoplastic oligodendrocytes,

but not in oligodendrogloma cells (24). Therefore, TPPP might act as a tumor suppressor in some malignancies by delaying the cell cycle and inhibiting proliferation, which would accord with our results.

In the future, liver parenchyma specimens taken from percutaneous liver biopsy for patients with chronic hepatitis or liver cirrhosis could predict the presence of HCC by evaluating TPPP expression. Furthermore, decreased TPPP expression in peripheral blood samples might help detect early-stage HCC in patients with chronic liver disease.

A possible limitation of this study is our use of only one CN sample for array analysis whereas we mixed 11 SN samples; however, we considered that we should not mix multiple cases in order to find an altered molecule in CN because a specific

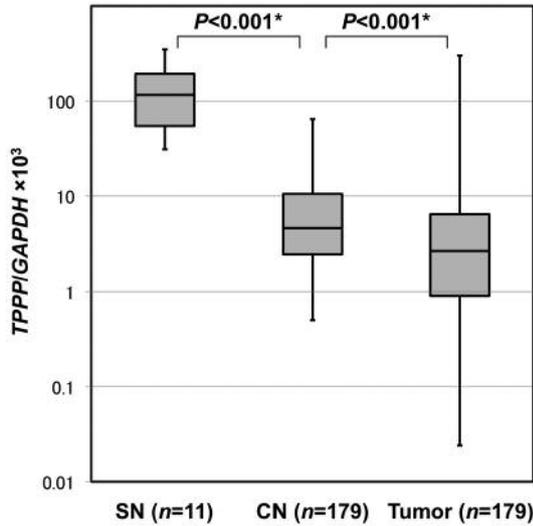


Figure 3. Result of real-time quantitative reverse transcription-polymerase chain reaction. Expression levels of tubulin polymerization-promoting protein (TPPP) mRNA, divided by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level, in specimens of super normal (SN, n=11) without hepatocellular carcinoma (HCC), corresponding normal (CN, n=179) of HCC and tumor tissues (n=179) of HCC determined by quantitative RT-PCR. TPPP expression was significantly lower in CN tissues than SN samples; with median TPPP $\times 10^3$ /GAPDH ratios for SN of 116 (range=31.0-348) vs. CN of 4.60 (range=0.5-64.3) ($p < 0.001$); and lower still in tumor tissues (median TPPP $\times 10^3$ /GAPDH ratio=2.63; range=0.0240-300; $p < 0.001$).

Table II. Methylation array analysis of tubulin polymerization-promoting protein (TPPP) in super normal (SN) and corresponding normal (CN) liver tissues.

Gene symbol	Sample	Methylation value (0-1)	Chromosomal location
TPPP	SN	0.384	Chr5p15.3
	CN	0.731	

abnormality is unlikely to exist in all cases. Conversely, in order to estimate the SN samples as controls without aberrance, we mixed 11 samples of normal liver tissue to erase tissue idiosyncrasy. We picked up the TPPP gene by array analysis, and then verified the result by specimens from 179 patients with HCC, which indicates that our result is widely applicable. As another limitation, we found no statistically significant relationship between promoter methylation and TPPP expression. However, TPPP transcripts were significantly lower among CN compared to SN, and among tumor tissues compared to CN (Figure 3), correspondingly, more frequent methylations were observed among CN (9.4%) compared to

Table III. Result of methylation-specific polymerase chain reaction (MSP) for super normal (SN), corresponding normal (CN) and tumorous liver tissues.

MSP	SN	CN	Tumor
+	0	17	51
-	11	162	128

SN vs. CN: $p = 0.6028$; SN vs. tumor: $p = 0.0381$; CN vs. tumor: $p < 0.001$.

Table IV. Correlation between tubulin polymerization-promoting protein (TPPP) expression indices and clinicopathological characteristics in 179 patients with hepatocellular carcinoma.

Characteristic	N	Median expression index	p-Value
Age (years)			
$\geq 65 / < 65$	85/94	0.433/0.584	0.252
Gender			
Male/female	152/27	0.492/0.614	0.376
HCV			
+/-	112/57	0.566/0.425	0.154
HBV			
+/-	40/129	0.365/0.560	0.120
Liver cirrhosis			
+/-	51/55	0.497/0.666	0.268
Tumor size			
≥ 5 cm / < 5 cm	61/94	0.373/0.595	0.221
Tumor number			
Solitary/multiple	109/50	0.614/0.368	0.071
Formation of capsule			
+/-	121/42	0.544/0.403	0.460
Growth form			
Expansive/infiltrative	134/21	0.539/0.338	0.154
Serosal infiltration			
+/-	31/98	0.360/0.472	0.560
Vascular invasion			
+/-	42/115	0.359/0.571	0.056
Differentiation			
Well/other	33/123	0.457/0.433	0.835
Well, moderate/poor	146/10	0.525/0.263	0.038
AFP (ng/ml)			
$\geq 20 / < 20$	88/78	0.420/0.676	0.012
Japan Stage			
I-II/III-IV	93/65	0.363/0.571	0.080

AFP, Alpha-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus. The expression index $[(TPPP_{tumor}) \times (GAPDH_{normal}) / (TPPP_{normal}) \times (GAPDH_{tumor})]$ was calculated for all 179 patients.

SN (0%), and among tumor tissues (28.4%) compared to CN (Table III). Thus, we assume that promoter methylation in TPPP is one of the mechanisms of down-regulation of this gene although it is not an exclusive means of regulation of mRNA expression.

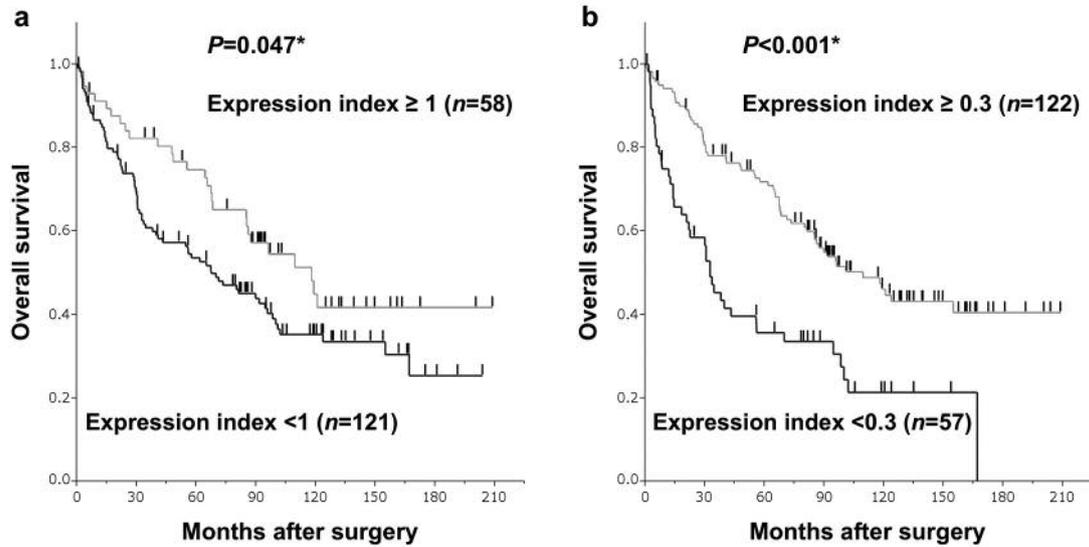


Figure 4. Overall survival rates of patients with hepatocellular carcinoma stratified by expression of tubulin polymerization-promoting protein (TPPP) mRNA. a: We classified the 179 cases according to whether TPPP expression index was higher (n=58) or lower (n=121) than 1.0. The TPPP expression index was calculated as [tumor tissue TPPP expression]/[adjacent normal tissue TPPP expression]. A log-rank test showed significant differences in overall survival rates (p=0.047). b: The 57 cases with very low TPPP expression indices (<0.3) had significantly worse prognoses than other patients (p<0.001).

Table V. Univariate and multivariate analyses of overall survival.

		N	Univariate analysis			Multivariate analysis		
			HR	95% CI	p-Value	HR	95% CI	p-Value
HCV	+	112	1.66	1.04-2.74	0.032	3.27	1.68-7.02	<0.001
	-	57	1					
Liver cirrhosis	+	51	1.69	1.03-2.79	0.036	1.58	0.892-2.86	0.115
	-	55	1					
Tumor number	Solitary	109	1					
	Multiple	50	1.72	1.10-2.64	0.016	1.08	0.619-1.86	0.763
Vascular invasion	+	42	2.54	1.59-3.96	<0.001	4.35	2.31-7.99	<0.001
	-	115	1					
Differentiation	Well, moderate	146	1					
	poor	10	2.39	1.06-4.67	0.036	1.7	0.550-4.36	0.327
AFP (ng/ml)	≥20	88	2	1.30-3.15	0.001	1.84	1.03-3.37	0.038
	<20	78	1					
Japan Stage	I-II	93	1					
	III-IV	65	1.61	1.05-2.47	0.028			
Index	≥1	58	1					
	<1	121	1.53	0.990-2.45	0.055			
Index	≥0.3	122	1					
	<0.3	57	2.49	1.64-3.76	<0.001	1.86	1.06-3.20	0.031

AFP, Alpha-fetoprotein; CI, confidence interval; HCV, hepatitis C virus; HR, hazard ratio. Index: Expression index $[(TPPP_{tumor}) \times (GAPDH_{normal}) / (TPPP_{normal}) \times (GAPDH_{tumor})]$.

In conclusion, TPPP expression in HCC background liver and tumor tissue is significantly less than in liver tissue of patients without HCC. These results imply that TPPP is a

tumor-suppressor gene for HCC when detected in background liver, and confirm the efficacy of multi-array analysis of HCC background liver.

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