Clinical Significance of Thrombospondin 1 Expression in Hepatocellular Carcinoma

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ABSTRACT

Purpose: Thrombospondin 1 (THBS 1) is a matricellular protein capable of modulating angiogenesis. However, the actual role of THBS 1 in angiogenesis and tumor progression remains controversial. Hepatocellular carcinoma (HCC) is a hypervascular tumor characterized by neovascularization. The significance of THBS 1 in HCC remains unknown. In this study, the significance of THBS 1 in HCC was evaluated by correlating its expression with clinicopathological data. The possible role of THBS 1 in the angiogenesis of HCC was also studied by correlating its expression with vascular endothelial growth factor (VEGF) expression.

Experimental Design: Sixty HCC patients were recruited in this study. THBS 1 and VEGF protein expression in tumorous livers were localized by immunohistochemical staining and quantified by ELISA. THBS 1 mRNA was quantified by quantitative reverse transcription-PCR.

Results: Immunohistochemical staining of THBS 1 was positive in HCC cells in 51.7% of patients and in stromal cells in 65% of patients. Tumor THBS 1 protein level was significantly correlated with its mRNA expression (P = 0.001) and was significantly correlated with tumor VEGF protein levels (P = 0.001). Its expression was significantly associated with the presence of venous invasion (P = 0.008) and advanced tumor stage (P = 0.049). High THBS 1 expression was also a prognostic marker of poor survival in HCC patients.

Conclusions: This study shows that high expression of THBS 1 is associated with tumor invasiveness and progression in HCC. THBS 1 appears to be a proangiogenic factor that stimulates angiogenesis in HCC in view of its positive correlation with VEGF expression.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly vascular tumor characterized by active neovascularization. It is the fifth most common malignancy worldwide (1) and the second leading cause of cancer death in Hong Kong (2). Hepatic resection is the best treatment offering a hope of cure for HCC. However, the long-term prognosis after resection remains unsatisfactory because of its propensity for vascular invasion and metastasis, leading to a high incidence of early postoperative recurrence in the liver remnant or distant sites (3).

It has previously been shown that angiogenesis makes an important contribution to the tumor growth, invasiveness, and metastatic potential of HCC (4–6). The degree of angiogenesis in a tumor is the net result reflecting complex interactions of various proangiogenic factors and antiangiogenic factors, secreted by the tumor cells, vascular endothelial cells, and infiltrating cells. Switching to angiogenic phenotype involves the up-regulation of proangiogenic factors, down-regulation of antiangiogenic factors, or both. Thus far, there are limited data on the role of various proangiogenic and antiangiogenic factors in HCC (6, 7). Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that plays a central role in tumor angiogenesis in many human cancers (8). It is also the most well-studied angiogenic factor in HCC and has been shown to play a central role in the angiogenesis and progression of HCC (6, 9).

Thrombospondin 1 (THBS 1) is one of the five members of the thrombospondin gene family, which includes THBS-1, -2, -3, -4, and -5 (10, 11). It is a matricellular protein synthesized and released by a variety of cell types into the extracellular matrix (12–14). The role of THBS 1 in angiogenesis and tumor progression remains controversial. THBS 1 has been considered an inhibitor of angiogenesis and tumor progression in some cancers (15–17). In contrast, it has also been considered a stimulator of both processes in other cancers (18–21). Some studies concluded that the actual role taken by THBS 1 is organ specific (22). Its effects may also depend on its concentration (20, 23), the type of domain being activated or available, and the type of receptors present on endothelial cells (24, 25).

Currently, the data on the role of THBS 1 in HCC are limited. The expression of THBS 1 in HCC cell lines and in clinical samples of HCC has been demonstrated (26–28). However, the relationship between THBS 1 expression and clinicopathological features of tumor invasiveness has not yet been studied in HCC. Hence, the significance of THBS 1 in HCC remains unknown. In this study, the expression of THBS 1 in HCC was investigated qualitatively and quantitatively. THBS 1 expression was correlated with clinicopathological data and...
prognosis. The possible role of THBS 1 in the angiogenesis of HCC would be elucidated by correlating its expression with VEGF expression.

MATERIALS AND METHODS

Patients and Specimens. Sixty patients who underwent resection of HCC between July 1998 and September 2001 were recruited in this study. The study protocol was approved by the Research Ethics Committee of The University of Hong Kong, and informed consent was obtained from all of the participants. There were 49 men and 11 women with a median age of 51.5 years (interquartile range, 45.0–63.5 years). The median tumor size was 7.1 cm (interquartile range, 4.0–10.8 cm). In addition, normal liver specimens from 10 healthy, living liver donors—size was 7.1 cm (interquartile range, 4.0–10.8 cm). There were 49 men and 11 women with a median age of 51.5 years (interquartile range, 45.0–63.5 years). In addition, normal liver specimens from 10 healthy, living liver donors were obtained immediately after resection of the tumors. One part of the tissues was snap-frozen in liquid nitrogen immediately and stored at −70°C. The other part was fixed in 10% buffered formalin and embedded in paraffin.

Immunohistochemical Staining for THBS 1 and Its Receptor. Cryostat sections, 8-µm thick, were fixed in −20°C acetone for 10 min. Staining procedure was performed using DAKO Envision Plus kit (DAKO Corp., Carpinteria, CA), following the manufacturer’s recommendations. Endogenous peroxidase activity was blocked by 0.03% hydrogen peroxide. Sections were incubated with 1:500 diluted mouse antihuman THBS 1 antibody (Sigma, Saint Louis, MO) for 30 min. Rabbit antimouse immunoglobulin conjugated with peroxidase labeled polymer was then applied for 30 min. The sections were developed in 3,3-diaminobenzidine and were counterstained with Mayer’s hematoxylin. Immunostaining was also performed using normal human serum in place of the primary antibody in serial sections of the samples as control.

Immunohistochemical staining for a THBS 1 receptor that has been shown to mediate the proangiogenic effect of THBS 1, the cysteine-serine-threonine-threonine-cysteine-glycine (CSVTG)-specific receptor (18), was also performed. Formalin-fixed 4-µm sections were deparaffinized, rehydrated, treated with 0.01 M citrate buffer in a microwave oven for 10 min for antigen retrieval. Staining procedure was performed using DAKO Envision Plus kit (DAKO Corp.), following the manufacturer’s recommendations. For the primary antibody, sections were incubated with 5 µg/ml mouse antihuman monoclonal antibody to CSVTGC-specific THBS 1 receptor (MC23.1; TSP Pharma, Phoenixville, PA) for 1 h at room temperature. The sections were developed in 3,3-diaminobenzidine and were counterstained with Mayer’s hematoxylin.

Immunohistochemical Staining for VEGF. Formalin-fixed, paraffin-embedded sections of 4 µm thick were deparaffinized and rehydrated. Antigen retrieval was done with microwave in 0.01 M citrate buffer. Staining procedure was performed using DAKO Envision Plus kit (DAKO Corp.), following the manufacturer’s recommendations. Endogenous peroxidase activity was blocked by 0.03% hydrogen peroxide. Sections were incubated with 1:25 diluted mouse antihuman VEGF monoclonal antibody (R&D Systems, Minneapolis, MN) for 2 h. Rabbit antimouse immunoglobulin conjugated with peroxidase-labeled polymer was then applied for 30 min. The sections were developed in 3,3-diaminobenzidine and counterstained with Mayer’s hematoxylin. Immunostaining was also performed using normal human serum in place of the primary antibody in serial sections of the samples as control.

Protein Extraction from Tumor Tissues. Cytosolic protein in frozen tissues was obtained by sonication small pieces of tissue samples in 1 ml of radioimmunoprecipitation assay buffer [25 mM Tris (pH 7.4), 0.15 mM KCl, 1% NP40, 5 mM EDTA, 0.5% sodium deoxycholate, and 0.1% SDS]. The lysate was spun down and supernatant was used for ELISA analysis of THBS 1 and VEGF.

ELISA Analysis of THBS 1. THBS 1 level in tumor cytosol and serum was quantified by ELISA. ELISA plates (MaxiSorp surface; Nalge Nunc International, Naperville, IL) were coated overnight with 1:500 diluted mouse antihuman monoclonal THBS 1 antibody (Sigma). After adding blocking buffer (1% BSA, 5% sucrose, and 0.05% sodium azide in PBS) for 1 h, duplicated samples were added for 2 h. Then, 1:500 diluted rabbit antihuman polyclonal THBS 1 antibody (Calbiochem, San Diego, CA) was added for 2 h. Subsequently, 1:4000 diluted horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (Zymed, San Francisco, CA) was added for 20 min. Tetramethylbenzidine in hydrogen peroxide (Pierce, Rockford, IL) was added for 20 min. The intensity of yellow color was evaluated at 450 nm after adding 2 N sulfuric acid. Control assays were performed in which an equivalent amount of irrelevant monoclonal antibody (antihuman Integrin αVβ3; Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used in place of the antihuman monoclonal THBS 1, or an equivalent amount of an irrelevant secondary antibody (rabbit antigoat IgG HRP; Zymed) was used in place of the HRP-conjugated goat antirabbit IgG. These controls had the same absorbance reading as a blank control, thus helping to establish the specificity of the THBS 1 assay.

ELISA Analysis of VEGF. VEGF in serum and tissue cytosolic samples was quantified by Quantikine human VEGF immunoassy (R&D Systems). Duplicated samples were added to the ELISA plate for 2 h. Then, HRP-conjugated polycyclonal VEGF-165 antibody was added for 2 h. Tetramethylbenzidine in hydrogen peroxide was added for 25 min. The intensity of yellow color was evaluated at 450 nm after adding 2 N sulfuric acid. The reliability and specificity of the assay has been described previously (9).

Real-Time Quantitative RT-PCR for THBS 1 mRNA Expression. Total RNA in frozen tissues was extracted using TRIZOL (Invitrogen) following the manufacturer’s recommendations. Total RNA was digested with DNase I (Invitrogen) and was used for first-strand cDNA reaction. The reaction mixture was composed for 0.5 µg of DNase I-treated RNA, 1× reverse transcriptase buffer, 4 mM dNTP mix, 1× random primer, and 2.5 units/µl multiScribe reverse transcriptase.

Real-time quantitative reverse transcription-PCR (RT-PCR) was performed using ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). The THBS
1 probe (5'-FAM-CTG CCA TCC GCA CTA A-MGB-3'), sense primer (5'-TGA CAA CAA CGT GGT GAA TGG-3'), and antisense primer (5'-ACA GGA GAT GCC GCA GAT G-3') were designed using the ABI PRISM Primer Express Software. Ribosomal 18s was used as an internal control. The quantitative PCR reactions contained 1× PCR buffer; 5.5 nM magnesium chloride; 200 μM dATP, dCTP, and dGTP; 400 μM dUTP; 200 nM THBS 1 sense and antisense primers, 50 nM THBS 1 FAM probe, 1× eukaryotic 18s rRNA minor groove binder (MGB) endogenous control, 0.025 units/μl AmpliTaq Gold Taq polymerase, and 5 μl of 1:60 fold diluted cDNA. The quantitative PCR condition was: 2 min at 50°C, 10 min at 95°C, thermal cycling proceeding with 40 cycles of 95°C for 15 s, and 60°C for 1 min. All reagents used for the RT-PCR reaction were purchased from Applied Biosystems.

The relative amount of THBS 1 mRNA, normalized to an internal control, ribosomal 18s (ΔCt), was defined as CThbs1T - CThbs1R, Ct was defined as the fractional cycle number at which the amount of amplified target reached a fixed threshold. The Ct value correlated with the amount of input target mRNA. A lower Ct value indicated a higher starting copy number. Huh 7 cell line was used to compare the relative amount of target transcripts in different samples and to adjust for the plate-to-plate variation in amplification efficiency.

Statistical Analysis. Continuous data were presented as median value (interquartile range). Mann-Whitney U and Wilcoxon rank tests were used to evaluate differences between unpaired and paired observations, respectively. Correlations were evaluated using the Spearman rank test. Overall survival rates of patients were computed by the Kaplan-Meier method and were compared by the log-rank test. Statistical significance was taken as P < 0.05. All statistical analyses were performed using the SPSS 11.0 statistical software package (SPSS Inc., Chicago, IL).

RESULTS

THBS 1, Its Receptor and VEGF Immunohistochemical Staining. In HCC tumor sections, specific THBS 1 immunostaining was found in the cytoplasm of HCC cells and stromal cells (Fig. 1). Immunostaining using normal human serum in place of the primary antibody was negative. THBS 1-positive HCC cells were found in 31 (51.7%) of the HCC patients. THBS 1-positive stromal cells were found in 39 (65%) of the HCC patients. Among 24 patients with positive THBS 1 staining in both HCC cells and stromal cells, most (87.5%) of the patients had stronger THBS 1 staining in stromal cells than in HCC cells. In the adjacent non-tumorous liver, THBS-1 immunostaining was found in the stromal cells in 17 (28.3%) of the patients, but THBS-1 staining was not observed in the hepatocytes in any case. Immunostaining for THBS-1 was negative in all 10 cases of normal liver specimens taken from healthy liver donors.

Positive staining of the CSVTCG receptor was observed in the tumor cells and stromal cells in the tumors (Fig. 2A). Positive staining of various intensity was found in the tumor cells in 57 (95%) of the patients and in the stromal cells in 53 (88.3%) of the patients, respectively. Some of the hepatocytes and endothelial cells in the non-tumorous liver also showed positive staining, but tumor tissue showed stronger staining than did non-tumorous tissue (Fig. 2). When the intensity of staining for THBS 1 and CSVTCG receptor in the tumor section per high-power field was scored by an automated computer image analyzer (MetaMorph Imaging System Version 3.0; Universal Imaging Corporation, West Chester, PA), there was a significant positive correlation between the staining of THBS 1 and CSVTCG receptor in the HCC specimens (r = 0.371; P = 0.015).

Specific VEGF immunostaining was found in the cytoplasm of HCC cells in 50 (83.3%) of the HCC patients (Fig. 3), but not in stromal cells. Immunostaining using normal human serum in place of the primary antibody was negative. In the adjacent non-tumorous liver, specific staining for VEGF was observed in the cytoplasm of hepatocytes in 47 (78.3%) of the cases, but the intensity of staining was weaker than that of the tumors in most cases. Immunostaining for VEGF was negative in all 10 cases of normal liver specimens taken from healthy liver donors.

THBS 1 and VEGF ELISA Analysis. The median cytosolic THBS 1 concentration in the tumor specimens was 8.54 ng/mg total protein (interquartile range, 4.51–16.34 ng/mg total protein). The median serum THBS 1 concentration was 17.28 μg/ml (interquartile range, 10.87–22.99 μg/ml). The serum
THBS 1 levels correlated significantly with platelet counts \((r = 0.509; \ P < 0.0001)\). However, there was no significant correlation between THBS 1 protein level in tumor and serum THBS 1 level \((r = 0.221; \ P = 0.098)\), or between THBS 1 protein level in tumor and serum THBS 1 level corrected by the platelet count, i.e., serum THBS 1 per platelet \((r = 0.049; \ P = 0.720)\).

The median cytosolic VEGF concentration in the tumor specimens was 33.00 pg/mg total protein (interquartile range, 14.35–111.76 pg/mg total protein). The median serum VEGF concentration was 263.05 pg/ml (interquartile range, 149.98–501.38 pg/ml). The serum VEGF-165 levels correlated significantly with platelet counts \((r = 0.544; \ P < 0.001)\). There was a significant correlation between tumor cytosolic VEGF levels and serum VEGF per platelet \((r = 0.461; \ P < 0.001)\).

In tumor cytosol, THBS 1 protein level was significantly correlated with VEGF protein level \((r = 0.409; \ P = 0.001)\). However, there was no significant correlation between serum THBS 1 and serum VEGF levels \((r = 0.251; \ P = 0.059)\).

**THBS 1 Quantitative RT-PCR Analysis.** To investigate whether the protein expression of THBS 1 was correlated with its mRNA expression, quantitative RT-PCR was performed to evaluate the mRNA expression of THBS 1. The median \(\Delta C_T\) of THBS 1 mRNA level in the tumor specimens was 12.67 (interquartile range, 11.21–13.69). THBS 1 protein level was significantly correlated with its mRNA expression level in tumor \((r = 0.537; \ P = 0.001)\).

**Correlation between THBS 1 Protein Expression and Clinicopathological Features.** The results of correlation between tumor THBS 1 expression and tumor pathological features are shown in Table 1. A significantly higher tumor THBS 1 protein expression level was associated with the presence of venous invasion \((P = 0.008)\) and advanced pathological tumor-node-metastasis (pTNM) tumor stages \((P = 0.049)\). There was a trend toward higher THBS 1 protein expression in the tumor with microsatellite nodules, but the difference was not statistically significant \((P = 0.051)\). However, tumor THBS 1 protein expression level was not significantly related to tumor size \((P = 0.499)\), the number of tumor nodules \((P = 0.690)\), or tumor encapsulation \((P = 0.594)\). There were no significant differences in serum THBS 1 levels between subgroups stratified by the tumor pathological parameters (Table 2). Tumor THBS 1 protein level was positively correlated with serum \(\alpha\) fetoprotein (AFP) level \((r = 0.374; \ P = 0.001)\). However, serum THBS protein level was not significantly correlated with serum AFP level \((r = 0.091, \ P = 0.511)\).

Because our previous study showed that a high-tumor VEGF expression level was associated with venous invasion and advanced tumor stage \((9)\), we analyzed the relationship between tumor THBS 1 expression level and these two pathological features of tumor aggressiveness after stratifying patients into those with high and those with low tumor cytosolic VEGF levels using the median level of 33.00 pg/mg total protein in the whole group as the cutoff level. High-tumor THBS 1 was significantly associated with venous invasion in both the high-tumor VEGF subgroup \((P = 0.038)\) and the low-tumor VEGF...
subgroup \((P = 0.027)\). There was a trend toward higher tumor THBS 1 in patients with more advanced-stage tumors in both subgroups of patients with high and low tumor VEGF levels, respectively, although the differences were not statistically significant \((P = 0.112 \text{ and } P = 0.093, \text{ respectively})\).

Correlation between Tumor THBS 1 Expression and Patients’ Survival. The prognostic value of THBS 1 on HCC patients’ overall survival was evaluated between patients with high and low tumor THBS 1 protein levels, stratified using 75 percentile as the cutoff value \((i.e., 16.34 \text{ ng/mg total protein})\) because the samples showed a skewed distribution of tumor THBS 1 levels \((30)\). It was observed that high tumor THBS 1 protein level was a significant prognostic factor of poor overall survival in HCC patients. HCC patients with high THBS 1 protein level in tumor cytosol had a significantly shorter overall survival \((1\text{-year 67%; 2\text{-year, 39%}) compared with those with low THBS 1 protein level in tumor cytosol \((1\text{-year, 84%; 2\text{-year, 73%; Fig. 4, } P = 0.014)\). However, there was not any significant relationship between serum THBS 1 and HCC patients’ survival \((P = 0.534)\).

**DISCUSSION**

In this study, THBS 1 immunohistochemical staining in HCC showed positive staining in both tumor cells and stromal cells in the majority of cases. The results were consistent with the previous study by Hayashi et al. \((26)\), who demonstrated that THBS 1 was synthesized by HCC cells, endothelial cells, and fibroblasts in the tumor, based on electron microscopy. In addition to THBS 1 synthesized in HCC cells, THBS 1 expression in

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### Table 1 Correlation between tumor THBS 1 protein expression and pathological features of hepatocellular carcinoma

<table>
<thead>
<tr>
<th>Pathological parameters</th>
<th>Median ng/mg total protein</th>
<th>Interquartile range ng/mg total protein</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
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<tr>
<td>(\leq 5\text{ cm (n = 23)})</td>
<td>7.26</td>
<td>3.57–13.68</td>
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<tr>
<td>(&gt;5\text{ cm (n = 37)})</td>
<td>8.80</td>
<td>4.54–23.71</td>
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<tr>
<td>Number of tumor nodules</td>
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<td></td>
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<tr>
<td>Solitary ((n = 38))</td>
<td>7.32</td>
<td>4.44–14.76</td>
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<tr>
<td>Multiple ((n = 22))</td>
<td>9.03</td>
<td>4.39–27.47</td>
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<tr>
<td>Tumor encapsulation</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Absent ((n = 41))</td>
<td>8.59</td>
<td>4.05–22.88</td>
<td>0.594</td>
</tr>
<tr>
<td>Present ((n = 19))</td>
<td>7.38</td>
<td>4.91–13.69</td>
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<tr>
<td>Microsatellite nodules</td>
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<td></td>
<td></td>
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<tr>
<td>Absent ((n = 42))</td>
<td>6.97</td>
<td>3.54–13.75</td>
<td>0.051</td>
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<tr>
<td>Present ((n = 18))</td>
<td>10.76</td>
<td>6.09–41.93</td>
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<tr>
<td>Venous invasion</td>
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<tr>
<td>Absent ((n = 33))</td>
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<td>3.45–13.12</td>
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<tr>
<td>Present ((n = 27))</td>
<td>12.08</td>
<td>6.24–39.36</td>
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<td>pTNM staging</td>
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<tr>
<td>Stages I and II ((n = 28))</td>
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<tr>
<td>Stages III and IV ((n = 32))</td>
<td>10.49</td>
<td>5.33–38.55</td>
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*THBS 1, thrombospondin 1; pTNM, pathological tumor-node-metastasis \((29)\).*

### Table 2 Correlation between serum THBS 1 protein expression and pathological features of hepatocellular carcinoma

<table>
<thead>
<tr>
<th>Pathological parameters</th>
<th>Median ng/mg total protein</th>
<th>Interquartile range ng/mg total protein</th>
<th>(P)</th>
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<tr>
<td>Tumor size</td>
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<td>(\leq 5\text{ cm (n = 23)})</td>
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<tr>
<td>Multiple ((n = 22))</td>
<td>16.31</td>
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<tr>
<td>Tumor encapsulation</td>
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<td></td>
<td></td>
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<tr>
<td>Absence ((n = 41))</td>
<td>17.53</td>
<td>11.16–22.82</td>
<td>0.903</td>
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<td>Presence ((n = 19))</td>
<td>16.47</td>
<td>9.60–26.71</td>
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<td>Microsatellite nodules</td>
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<td>Absence ((n = 42))</td>
<td>18.20</td>
<td>10.25–23.07</td>
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<td>Presence ((n = 18))</td>
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<td>Venous invasion</td>
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<tr>
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<td>0.665</td>
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<tr>
<td>Presence ((n = 26))</td>
<td>16.93</td>
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<td>Stages I and II ((n = 28))</td>
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<td>Stages III and IV ((n = 32))</td>
<td>17.00</td>
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*THBS 1, thrombospondin 1; pTNM, pathological tumor-node-metastasis \((29)\).*
stromal cells surrounding the tumor cells may provide favorable conditions for tumor invasion and angiogenesis (26). In this study, we observed that THBS 1 staining was stronger in the stromal cells than in the HCC cells in the majority of patients with positive staining for both. This is in accordance with previous studies in other cancers (31). THBS 1 staining was also observed in the stromal cells of nontumor liver in a small proportion (28.3%) of patients, whereas no staining for THBS 1 was observed in the normal liver specimens. The nontumorous liver in HCC patients is usually affected by chronic hepatitis or fibrosis, and THBS 1 may be expressed in such conditions. In fact, Hayashi et al. have also demonstrated the staining for THBS 1 in the fibroblasts of the surrounding nonmalignant liver in HCC patients (26). Although the expression of THBS 1 in HCC and surrounding liver has been previously reported (26), its relationship with angiogenesis in HCC has not been reported in the literature. This study evaluated the possible role of THBS 1 in the angiogenesis in HCC by correlating its expression with that of VEGF. Furthermore, we also investigated the clinicopathological correlation and prognostic influence of tumor THBS 1 expression in HCC, which had not been studied in the previous report on THBS 1 expression in HCC (26).

VEGF, a specific potent proangiogenic factor, is a key mediator in the angiogenesis in most cancers and is a mediator involved in the angiogenic effects of many other angiogenic factors (8). Immunohistochemical staining showed that VEGF was expressed in 83.3% of HCC tumor specimens. VEGF was also expressed in the nontumorous liver in the majority of cases, although the intensity of staining was weaker than that in the tumors. VEGF may play a role in regulating angiogenesis in cirrhotic liver, which is common in patients with HCC (32). The cytosol levels of THBS 1 and VEGF protein in the tumor were significantly correlated, suggesting that THBS 1 may act as a proangiogenic factor rather than an antiangiogenic factor in HCC. THBS 1 could enhance angiogenesis by facilitating the extracellular matrix degradation (33). This is important in the process of angiogenesis because a suitable microenvironment is created for endothelial cell proliferation, migration, and differentiation. As an adhesive protein, THBS 1 can also facilitate angiogenesis by enhancing the adhesion of endothelial cells after sprouting. This is the first study suggesting a proangiogenic role of THBS 1 in HCC. THBS 1 has already been reported to be an angiogenesis stimulator in pancreas cancer (20), breast cancer (18), cutaneous melanomas (21), and colorectal cancer (19). By targeting the factors regulating its expression, antiangiogenic therapy for therapeutic intervention can be developed to inhibit tumor angiogenesis and halt tumor progression.

THBS 1 was originally discovered as a secretory product of platelets, sequestered in the α-granules (34). It is released into the circulation on platelet activation. In this study, it was observed that serum THBS 1 levels correlated significantly with platelet counts. Hence, the major source of serum THBS 1 may be contributed by activated platelets. It has been shown in a previous study by the authors’ group (9) that serum VEGF could be used to estimate tumor VEGF expression. It is advantageous in a way that the VEGF expression in the tumor can be estimated without the requirement of a biopsy or surgical specimen. Hence, the possible significance of serum THBS 1 has also been investigated in this study. However, neither serum THBS 1 nor platelet load of serum THBS 1 (serum THBS 1 per platelet) correlated with tumor THBS 1 expression. Furthermore, serum THBS 1 did not correlate with any of the pathological features of tumor invasiveness or serum VEGF level. Thus, circulating THBS 1 level cannot be used as a surrogate marker for evaluating tumor THBS 1 expression or tumor status in HCC patients.

Analysis of THBS 1 protein expression in relation to clinicopathological features showed that high THBS 1 in the tumor was significantly associated with venous invasion and advanced pTNM tumor stage. There was also a trend toward a higher THBS 1 protein expression in the tumors with microsatellite nodules, although the difference was not statistically significant. These findings suggest an important role of THBS 1 in tumor invasiveness and progression of HCC. Venous invasion is one of the most important pathological features that lead to postoperative tumor recurrence after resection of HCC (3, 7). The association between high THBS 1 in venous invasion or advancing tumor stage is in line with its proangiogenic role in angiogenesis. Our previous study showed that a high tumor expression level of VEGF was significantly associated with venous invasion and advanced tumor stage. It is possible that the association between high tumor THBS 1 level and tumor progression may be partly mediated by VEGF, because there was a significant correlation between tumor THBS 1 and VEGF levels. However, when we performed a stratified analysis of the association between THBS 1 and venous invasion or tumor stage in subgroups of patients with high and low tumor VEGF levels, respectively, we found a significant relationship between tumor THBS 1 level and venous invasion even in patients with low tumor VEGF levels. This suggests that THBS 1 may have a role in the tumor progression of HCC separate from that of VEGF. There was a trend of association between high tumor THBS 1 level and advanced tumor stage in both subgroups of high and low tumor VEGF levels, although the difference was not stas-
tically significant, which might be related to the small sample size after stratifying the patients into two subgroups. This study also revealed a positive correlation between tumor THBS 1 protein level and serum AFP level. Some studies suggest that high serum AFP level is associated with a more aggressive tumor and increased risk of recurrence or metastasis (7, 35). The finding of a positive correlation between tumor THBS 1 and serum AFP further supported a role of THBS 1 in the aggressiveness of HCC.

Not many studies have been performed on the association of THBS 1 expression and the survival of cancer patients. In a study of THBS 1 protein expression in invasive transitional cell carcinoma of the bladder, THBS 1 expression was a significant prognostic predictor of overall survival (15). In that study, however, high tumor THBS 1 level resulted in a higher overall survival compared with low tumor THBS 1 level. In another study of THBS 1 protein expression in melanoma, high expression of THBS 1 resulted in an aggressive tumor behavior, including decreased overall survival and the presence of venous invasion (21). In agreement with the latter study, our results showed that high THBS 1 expression was an unfavorable prognostic factor in HCC. This can be attributed to the observation that a significantly higher THBS 1 protein expression was associated significantly with the presence of venous invasion and advancing pTNM tumor stage, both of which are the most important pathological predictors of poor survival after resection of HCC (3, 7). Additional studies on larger patient samples are required to validate the prognostic value of tumor THBS 1 expression.

In previous studies, it has been speculated that the actual role of THBS 1 in angiogenesis depends on the nature and number of THBS 1 receptors, which vary in different tumors (36). Its effects may also depend on the type of domain being activated and type of receptors present on endothelial cells. For instance, when the CSVTCG domain of THBS 1 binds to the phosphorylated state of CD36, THBS 1 acts as an antiangiogenic factor (37, 38). However, when the same domain binds to CSVTCG receptor, THBS 1 acts as a proangiogenic factor (18, 23). In this study, we also evaluated the expression of the CSVTCG receptor of THBS 1 in the tumor specimens by immunostaining. In line with the findings of studies of the receptor in other cancers (18, 39), CSVTCG receptor was expressed by both the cancer cells and stromal cells in most cases of HCC. We found that the intensity of CSVTCG receptor staining in the tumor was higher than that in the nontumorous liver, and there was a positive correlation between the intensity of THBS 1 and its CSVTCG receptor staining in the tumor. Our findings suggest that THBS 1 may promote tumor progression via CSVTCG receptor, which has been shown to mediate the effect of THBS 1 on tumor progression in other cancers such as breast carcinoma and gastric carcinoma (18, 23, 39). It has also been reported in a previous study that THBS 1 promoted venous invasion through up-regulation of urokinase plasminogen activator and plasmin (40). In the plasminogen-plasmin system, urokinase plasminogen activator generates plasmin from plasminogen. Plasmin, a protease with broad specificity, is able to degrade most extracellular matrix proteins and activate a number of matrix metalloproteinases, including matrix metalloproteinase 9. It is also possible that THBS 1 modulates venous invasion via such a pathway. The exact mechanism by which THBS 1 protein modulates tumor invasiveness in HCC still needs to be clarified.

In conclusion, this is the first study demonstrating the clinical significance of THBS 1 in HCC. Higher THBS 1 expression is associated with the presence of venous invasion and advancing pTNM tumor staging, and high THBS 1 is also a significant prognostic marker of poor survival in HCC patients. This study showed a positive correlation of THBS 1 and VEGF expression in HCC and suggested that THBS 1 may enhance tumor invasion and progression through a proangiogenic role in HCC. However, additional studies are needed to clarify the mechanism by which THBS 1 is involved in the progression of HCC, and its exact role in the regulation of angiogenesis in HCC.

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Clinical Significance of Thrombospondin 1 Expression in Hepatocellular Carcinoma

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