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Thrombospondin 2 expression is correlated with inhibition of angiogenesis and metastasis of colon cancer

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Summary Two subtypes of thrombospondin (TSP-1 and TSP-2) have inhibitory roles in angiogenesis in vitro, although the biological significance of these TSP isoforms has not been determined in vivo. We examined *TSP-1* and *TSP-2* gene expression by reverse transcription polymerase chain reaction (RT-PCR) analysis in 61 colon cancers. Thirty-eight of these 61 colon cancers were positive for *TSP-2* expression and showed hepatic metastasis at a significantly lower incidence than those without *TSP-2* expression ($P = 0.02$). *TSP-2* expression was significantly associated with M0 stage in these colon cancers ($P = 0.03$), whereas *TSP-1* expression showed no apparent correlation with these factors. The colon cancer patients with *TSP-2* expression showed a significantly low frequency of liver metastasis correlated with the cell-associated isoform of vascular endothelial growth factor (VEGF-189) ($P = 0.0006$). Vascularity was estimated by CD34 staining, and TSP-2(–)/VEGF-189(+) colon cancers showed significantly increased vessel counts and density in the stroma ($P < 0.0001$). TSP-2(–)/VEGF-189(+) colon cancer patients also showed significantly poorer prognosis compared with those with TSP-2(+) / VEGF-189(–) ($P = 0.0014$). These results suggest that colon cancer metastasis is critically determined by angiogenesis resulting from the balance between the angioinhibitory factor TSP-2 and angiogenic factor VEGF-189.

Keywords: vascular endothelial growth factor; thrombospondin; colon cancer

The prognosis of colon cancer is correlated with the presence or absence of distant metastasis. Cancer metastasis requires stromal angiogenesis including proliferation of endothelial cells and migration through extracellular matrix barriers. Angiogenesis depends on the local balance between various molecules that induce and inhibit neovascularization. In the microenvironment around successful tumours, this balance shifts from neutral to angiogenic conditions. Angiogenic factors secreted by tumours induce a vigorous angiogenic response overcoming potent inhibitors.

Thrombospondin (TSP) is a high-molecular-weight multifunctional glycoprotein which was first described as a product of platelets (Baenziger et al, 1972), released from the alpha granules in response to activation of thrombin (Lawler et al, 1986). TSP is not only one of the components of the extracellular matrix (ECM) in a wide variety of tissues (O'Shea et al, 1988; O'Shea et al, 1990), but also contains binding sites for a number of ECM and cell-associated molecules (Taraboletti et al, 1987; Sage et al, 1991). TSP is synthesized and secreted by various types of cells, i.e. fibroblasts (Jaffe et al, 1983), smooth muscle cells (Majack et al, 1987), monocytes and macrophages (Jaffe et al, 1985), osteoblasts (Gehron et al, 1989) and various neoplastic cells (Roberts et al, 1987; Zabrenetzky et al, 1994; Qian et al, 1996). TSP induces platelet aggregation (Legrand et al, 1992) and inhibits angiogenesis (Iruela et al, 1991; Weinstat et al, 1994). There are five subtypes of TSPs, namely TSP-1, TSP-2 (Labell et al, 1993), TSP-3 (Vos et al, 1992), TSP-4 (Lawler et al, 1993) and cartilage oligomeric matrix protein (Oldberg et al, 1992). Of the five structurally different TSPs, TSP-1 and TSP-2 show similarities in their molecular architecture and modulatory effects on angiogenesis (Bornstein et al, 1991; Bornstein et al, 1992; Laherty et al, 1992). Recently, TSP-1 has been implicated in progression in melanoma, lung cancer and breast cancer cell lines (Zabrenetzky et al, 1994). Transfection of TSP-1 cDNA into a human breast carcinoma cell line reduced primary tumour growth, metastatic potential and angiogenesis (Weinstat et al, 1994). TSP-1 expression inhibits angiogenesis in human glioblastoma cell lines (Hsu et al, 1996). TSP-2 has not been well characterized, although there have been a few reports of its cell biological behaviour and expression in breast cancer (Laherty et al, 1992; Bertin et al, 1997).

Vascular endothelial growth factor (VEGF) is an inducer of angiogenesis (Connolly et al, 1989; Takahashi et al, 1995; Warren et al, 1995). We demonstrated that expression of the cell-associated isoform VEGF-189 was significantly correlated with metastasis and prognosis in colon cancer (Tokunaga et al, 1998). TSP-1 and TSP-2 are potent antiangiogenic factors in colon cancer. In this study, we evaluated *TSP-1* and *TSP-2* expression in colon cancer as antiangiogenic factors, and discuss here the local balance of these factors in association with the angiogenic factor VEGF.

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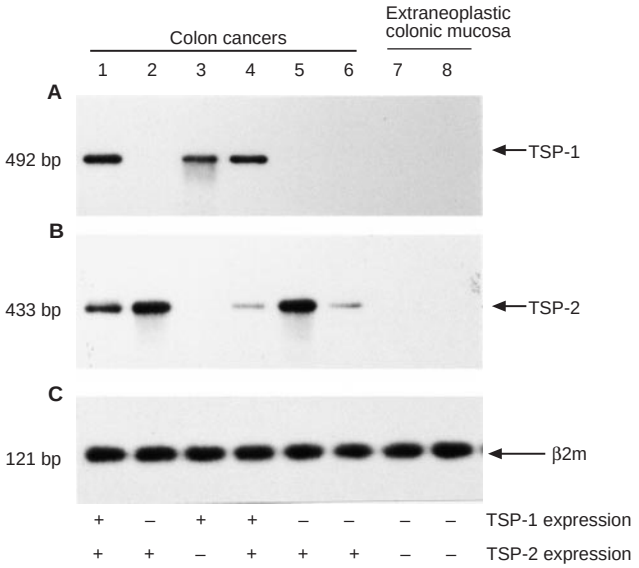

Figure 1 Examples of *TSP-1* and *TSP-2* gene expression in colon cancers and extraneoplastic specimens: *lanes 1–6, colon cancer specimens; lanes 7 and 8, extraneoplastic colonic mucosal specimens.* (**A**) *TSP-1* expression was detected by RT-PCR with primers Th1-S and Th1-A, showing a 492-bp specific fragment. (**B**) *TSP-2* expression was detected by RT-PCR with primers Th2-S and Th2-A, showing a 433-bp specific fragment. (**C**) β2m gene expression was evaluated to qualify RNA samples

MATERIALS AND METHODS

Subjects and tissue samples

The subjects in this study were 61 patients (27 women and 34 men, mean age 61.4) with colon cancer (58 adenocarcinoma, three mucinous carcinoma) who underwent surgical resection between October 1989 and October 1991 at Tokai University Hospital, Kanagawa, Japan. All patients were evaluated by TNM score. Surgical specimens were rapidly frozen and stored at –80°C until analyses.

Expression of *TSP-1* and *TSP-2* genes

We evaluated TSP mRNA expression by reverse transcription polymerase chain reaction (RT-PCR) using the following primers: for TSP-1, Th1-S, 5′-ACCGCATTCCAGAGTCTGGC-′3; Th1-A, 5′-ATGGGGACGTCCAACTCAGC-′3; and for TSP-2, Th2-S, 5′-CTGTGTCAACACTCAGCCTGGC-′3; Th2-A, 5′-TCCTTCT-CATCGGTCACACCG-′3. Reverse transcription was performed at 42°C for 60 min (1 μg total cellular RNA; 100 pM random primers, 40 U reverse transcriptase, Gibco-BRL). DNA fragments were amplified by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min using a Gene Amp PCR System 9600 (Perkin Elmer) and *Taq* DNA polymerase (1.3 U Toyobo, Japan). Blots (Zeta-Probe, Bio-Rad) were hybridized with photochemically labelled TSP-1- or TSP-2-specific cDNA probe (ECL; Amersham) and exposed to Kodak AR film. *TSP* gene expression was also estimated by Northern blotting analysis with total cellular RNA (15 μg, GeneScreen Plus, New England Nuclear). The quality of the RNA was estimated by RT-PCR for β2-microglobulin.

Table 1 Univariate analysis of the associations between *TSP* gene expression and tumour characteristics

	TSP-1		*P*-value	TSP-2		*P*-value
	+	–		+	–	
v-factor			0.498			0.711
v1+ ≥	14	17		20	11	
v2+ ≤	11	19		18	12	
ly-factor			1			0.739
ly1+≥	20	29		31	18	
ly2+≤	5	7		7	5	
T-staging			0.471			0.739
T0–T2	6	6		7	5	
T3, T4	19	30		31	18	
N-staging			0.131			0.561
N0	16	16		21	11	
N–N3	9	20		17	12	
M-staging			0.554			0.03[a]
M0	19	25		31	13	
M1	6	11		7	10	
Liver metastasis			0.344			0.016[a]
Yes	5	11		6	10	
No	20	25		32	13	
K-*ras* mutation			0.271			0.242
Yes	9	18		19	8	
No	16	18		19	15	

[a]TSP-2 expression was inversely correlated with M0 stage and liver metastasis of colon cancer ($P < 0.05$, χ^2 test). The degree of venous invasion (v-factor) was classified into four groups as follows: v0, no venous invasion; v1+, minimal venous invasion, i.e. one or two foci of venous invasion in the histological sections; v2+, moderate venous invasion, i.e. three or four foci of venous invasion; and v3+, severe venous invasion, i.e. more than five invasion foci. Also, the degree of lymphatic invasion (ly-factor); ly1+, mild lymphatic invasion; ly2+, moderate lymphatic invasion; and ly3+, severe lymphatic invasion.

Vascularization in colon cancer

Formalin-fixed, paraffin-embedded sections of the tumour tissue were examined immunohistochemically with mouse anti-human CD34 monoclonal antibody (NCL-end, Novo Castra). After blockage of endogenous peroxidase activity (methylalcohol, 3% H_2O_2) and non-specific binding (10% normal goat serum), specimens were incubated with anti-CD34 antibody (1:20) at room temperature for 60 min. Sections were serially incubated with biotin-labelled anti-mouse IgG (Nichirei, Tokyo, Japan) and horseradish peroxidase – conjugated streptavidin (Nichirei, Tokyo, Japan). Reaction products were visualized with 3, 3′-diaminobenzidine. Light microscopy was used to identify two regions within or immediately adjacent to the cancer containing the highest numbers of vessels. The microvessel counts and densities were evaluated at × 200 magnification (× 20 objective and × 10 ocular, 0.739 mm^2 per field) using a computerized image analyser (Interactive Build Analysis System, Zeiss).

Statistical analysis

Differences in survival between subgroups of patients were compared with the log-rank test, and survival curves were plotted according to the method of Kaplan and Meier. The χ^2 test or Fisher's exact test was applied for comparisons between group

Table 2 *TSP* gene expression and VEGF mRNA isoform patterns

TSP expression	VEGF type 1/2[a]	VEGF type 3[a]
TSP-1 (+)	10	15
TSP-1 (–)	19	17
TSP-2 (+)	18	20
TSP-2 (–)	11	12

[a]Types 1/2 expressed VEGF-121 and/or VEGF-165. Type 3 expressed VEGF-121, –165 and –189.

Table 3 Associations between *TSP-2* gene expression linked with VEGF mRNA isoform patterns and tumour characteristics

	VEGF type 1/2 TSP-2 (+)	VEGF type 3 TSP-2 (–)	*P*-value
v-factor			0.0236[a]
v1+ ≥	13	3	
v2+ ≤	5	9	
ly-factor			0.4181
ly1+ ≥	14	7	
ly2+ ≤	4	5	
Vessel counts	37 ± 16	77 ± 23	< 0.0001[b]
Vessel density	2.9 ± 1.2	6.8 ± 2.0	< 0.0001[b]
T-staging			0.6599
T0–T2	3	3	
T3, T4	15	9	
N-staging			0.3915
N0, N1	15	8	
N2, N3	3	4	
M-staging			0.0012[c]
M0	16	3	
M1	2	9	
Liver metastasis			0.0006[c]
Yes	1	8	
No	17	4	

Significance of differences was evaluated by χ^2 test[a], ANOVA[b], Fisher's test[c].

frequencies. The statistical significance of differences in mean vessel counts and density among the groups were examined by ANOVA. Multiple comparisons were performed by the Bonferroni method.

RESULTS

TSP-1 and *TSP-2* gene expression

Four of 61 colon cancer specimens expressed *TSP-1* but not *TSP-2*, whereas 17 expressed *TSP-2* but not *TSP-1* as shown by RT-PCR analysis (examples are shown in Figure 1A and B). Twenty-one of the 61 colon cancers coexpressed both *TSP-1* and *TSP-2* genes. The extraneoplastic colonic mucosal specimens demonstrated little *TSP* gene expression. *TSP-1* expression was detected in 7 out of 36 extraneoplastic colonic mucosal specimens by RT-PCR, although *TSP-1* gene expression was not detectable in the extraneoplastic tissue by Northern blotting. Moreover, *TSP-2* gene expression of colon cancer specimens (38 out of 61) was found at a significantly higher incidence than in extraneoplastic tissue (3 out of 36). Northern blotting analyses confirmed faint

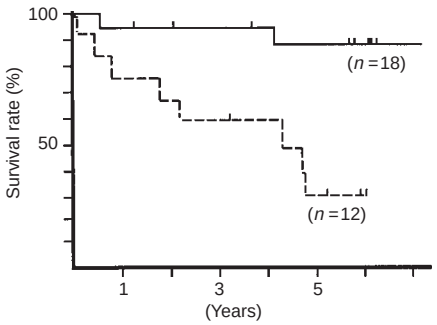

Figure 2 The prognosis of colon cancer patients is shown on a Kaplan–Meier plot (generalized log-rank test). The prognosis of patients with expression of *TSP-2* and type 1 or 2 VEGF isoform pattern [TSP-2(+)/VEGF-189(–), solid line] was significantly better than that of patients with type 3 VEGF isoform pattern and lacking *TSP-2* expression [TSP-2(–)/VEGF-189(+), broken line] ($P = 0.0014$)

TSP-2 gene expression in 12 out of 38 colon cancer specimens with definite *TSP-2* expression by RT-PCR, whereas the levels of expression were varied (data not shown). Southern blotting analyses showed neither amplification nor rearrangement of the *TSP-2* gene (data not shown).

Correlation between *TSP* expression and distant metastasis

The patients with colon cancers showing *TSP-2* expression had a significantly lower incidence (6 out of 38, 15.8%) of liver metastasis than those (10 out of 23, 43.5%) without TSP-2 expression ($P = 0.02$, χ^2 test, Table 1). Six out of the 16 (37.5%) patients with hepatic metastatic lesions showed *TSP-2* expression, whereas 32 out of the 45 patients (71.1%) without liver metastasis showed *TSP-2* expression. *TSP-2* expression was inversely correlated with the distant metastasis (M1 stage) of colon cancer ($P = 0.03$, χ^2-test, Table 1). *TSP-2* expression was not correlated with tumour size (Tx stage) or nodal involvement (Nx stage), and expression of *TSP-1* was not correlated with any of the clinical characteristics examined (Table 1).

Association between *TSP* gene expression and VEGF isoform

Previously, we reported that VEGF mRNA isoform expression patterns in colon cancer could be divided into three types: type 1, VEGF-121; type 2, VEGF-121/165; and type 3, VEGF-121/165/189 (Tokunaga et al, 1998). Three out of the 61 colon cancers showed type 1 expression. Twenty-six out of the 61 patients showed type 2 expression, and the remaining 32 showed the type 3 pattern. None of the tumours showed VEGF-206 expression. Twelve out of the 16 (75.0%) patients with hepatic metastatic lesions showed the type 3 pattern, whereas 20 out of the 45 patients (44.4%) without liver metastasis showed this isoform expression pattern. We analysed the correlation between *TSP* gene expression and VEGF mRNA isoform pattern (Table 2). Ten out of 25 colon cancers expressing *TSP-1* showed types 1 and 2 VEGF isoform patterns. Fifteen of those expressing *TSP-1* showed type 3 VEGF isoform pattern. Eighteen out of 38 colon cancers expressing the *TSP-2* gene showed types 1 and 2 VEGF isoform patterns, whereas 20 of those expressing *TSP-2* showed the type 3

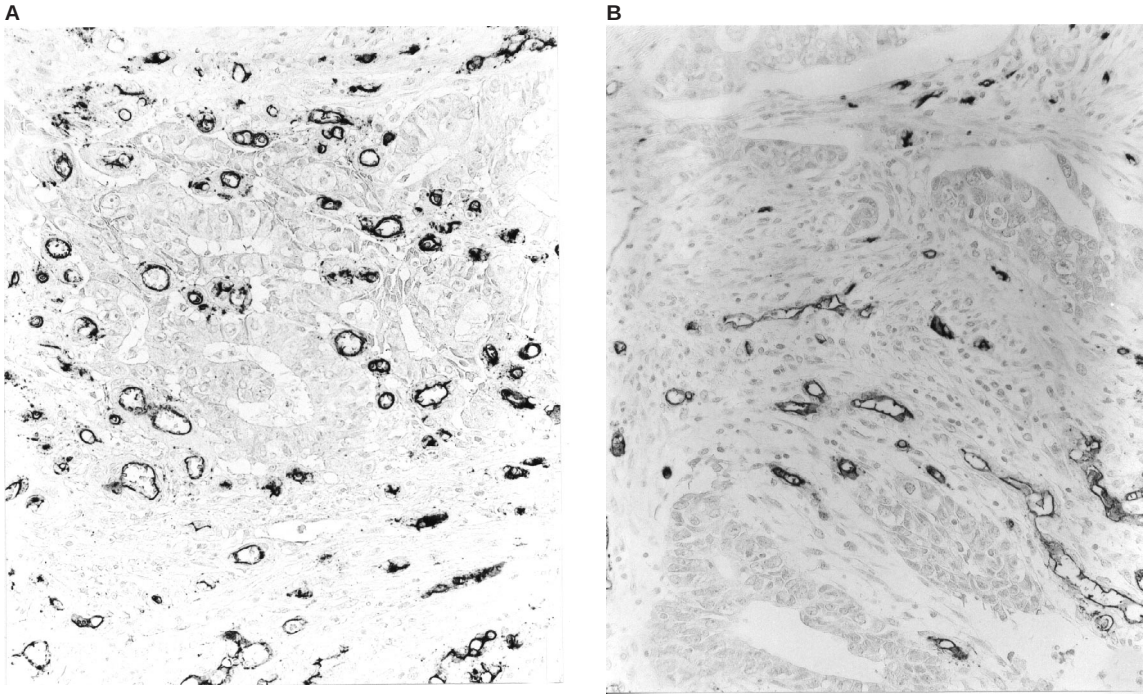

Figure 3 Vascularization in colon cancer was demonstrated by immunostaining for CD34. (**A**) Colon cancer [TSP-2(–)/VEGF-189(+)] showed significantly increased vascular density. (**B**) Colon cancer [TSP-2(+)/VEGF-189(–)] showed moderate vascular density (× 200)

pattern. The type 3 VEGF isoform pattern was observed in 12 colon cancers without *TSP-2* gene expression. There was no significant correlation between *TSP* gene expression and VEGF isoform expression pattern (Table 2). Patients with colon cancer expressing *TSP-2* mRNA without VEGF-189 expression showed hepatic metastasis at a significantly lower incidence [TSP-2(+)/V189(–) group; 1 out of 18, 5.6%] than those with colon cancer expressing no TSP-2 with VEGF-189 expression [TSP-2(–)/V189(+) group; 8 out of 12, 75.0%] ($P = 0.0006$, Fisher's test, Table 3). TSP-2(+)/V189(–) group patients showed significantly better prognosis ($P = 0.0014$, log rank test, Figure 2) than the TSP-2(–)/V189(+) group.

Vascularization and *TSP* expression

The mean vessel count and density in all colon cancers examined were 58.4 ± 27.7 per × 200 fields (range 16–153) and 4.7% ± 2.8% per × 200 fields (range 1.1–12.1) respectively. The mean vessel count in TSP-2(+)/V189(–) group was 36.5 ± 15.9 (range 16–70). That of the TSP-2(–)/V189(+) group was 77.1 ± 22.6 per × 200 fields (range 45–146). However, those of TSP-2(+)/V189(+) and TSP-2(–)/V189(–)group were 62.5 ± 38.7 and 52.5 ± 41.4 respectively. The mean vessel density in TSP-2(+)/V189(–) group was 2.9% ± 1.2% per × 200 fields (range 1.1–5.2), whereas that in the TSP-2(–)/V189(+) group was 6.8% ± 2.0% per × 200 fields (range 2.9–12.1). Those of TSP-2(+)/V189(+) and TSP-2(–)/V189(–) groups were 5.0 ± 4.3 and 4.5 ± 3.6 respectively. Significant differences were found among the four groups in vessel counts ($P < 0.0001$, ANOVA test) and vessel density ($P < 0.0001$, ANOVA test). The most significant difference was found between the TSP-2(+)/V189(–) and TSP-2(–)/V189(+) groups ($P < 0.0001$, Bonferroni test) (Figure 3, Table 3). Patients with colon cancer expressing VEGF-189 without *TSP-2* expression showed involvement of veins at a significantly higher incidence ($P = 0.0236$, Fisher's test, Table 3). There were no correlations between *TSP-1* or *TSP-2* mRNA expression and any histological feature of colon cancer examined (Table 1).

DISCUSSION

Angiogenesis in the stroma is a major factor contributing to distant metastasis of colon cancers. Stromal angiogenesis control is based on the balance between various angiogenic cytokines and angioinhibitory factors. Thrombospondin (TSP) is an antiangiogenic factor (Iruela et al, 1991; Weinstat et al, 1994; Zabrenetzky et al, 1994; Volpert et al, 1995; Hsu et al, 1996). We detected TSP transcripts in 69% of primary human colon cancer specimens. It is possible that variable amounts of stromal RNA were copurified from the tumours in these bulk studies. *TSP-1* expression was detected in seven specimens of 36 extraneoplastic colonic mucosa by RT-PCR, whereas none of these were detected by Northern blotting. *TSP-2* expression was detected in 3 out of 36 extraneoplastic colonic mucosal specimens by RT-PCR. This was a significantly lower frequency than that in cancer specimens. Although it is still unclear whether the neoplastic cells or stromal cells predominantly express *TSP-1* or *TSP-2*, cancer tissue specimens showed expression of these genes at increased frequency and intensity. It was reported that *TSP* expression was more intense in the stromal tissues within and immediately adjacent to tumours (Clezardin et al, 1993; Grossfeld et al, 1996), whereas TSP was localized to some normal tissues including peritubular connective tissue of the kidney, the basement membrane regions beneath glandular epithelium in the lung, epidermal–dermal junctions, and the interstitium in skeletal muscles (Wight et al, 1985). The results presented in this study supported the view that TSP is more predominantly expressed in colon cancer than in the extraneoplastic tissue.

Five different subtypes of human TSP have been identified. Among the TSP subtypes, TSP-2 showed considerable protein sequence similarity to that of TSP-1 which is known to be functionally critical. TSP-1 and TSP-2 are clearly distinct gene products (Labell et al, 1993), although both modulate angiogenesis (Volpert et al, 1995). TSP-2 blocks the migration of capillary endothelial cells evoked by a variety of inducers, and inhibits neovascularization in the rat cornea (Panetti et al, 1997). Recombinant murine TSP-2 inhibited bovine aortic endothelial cell proliferation at doses similar to those at which TSP-1 also showed this effect (Volpert et al, 1995). In various neoplastic cell lines, the level of *TSP-1* expression showed an inverse correlation with malignant progression (Weinstat et al, 1994; Zabrenetzky et al, 1994; Hsu et al, 1996). Recently, tumour progression and angiogenesis in bladder cancer were reported to be associated with *TSP-1* expression (Grossfeld et al, 1997). It has been postulated that TSP-1 modulates tumour progression through its inhibitory effect on tumour angiogenesis. However, there have been few reports concerning *TSP-2* expression encoded by genes other than *TSP-1* and functions in neoplasms including colon cancer. Two specific primers were used to confirm *TSP-1* or *TSP-2* gene expression accurately. Putative misamplified RT-PCR products do not show confusing cDNA fragment lengths (within 200 bp difference) compared with the TSP-1 or TSP-2-specific fragments, even if the primers misreact because of the 65% sequence homology. Moreover, we also confirmed that TSP-1/TSP-2-specific probes did not crossreact with each other under the conditions used for Southern blotting. The RT-PCR procedures used in this study were sufficient to specifically detect TSP-1 and TSP-2 mRNA. The results of our study demonstrated that TSP-2 was more predominantly expressed in colon cancer than TSP-1. We showed that the *TSP-2* expression was inversely and significantly correlated with liver metastasis and M1 stage in colon cancer, whereas *TSP-1* expression did not show any such correlations. These results suggested that TSP-2 is a major inhibitory factor against distant metastasis of colon cancer.

TSP-2 expression exhibited a significant correlation with stromal vessel counts and density when they were analysed with expression of the cell-associated isoform VEGF-189. Those colon cancers expressing VEGF-189 without TSP-2 [TSP-2(–)/VEGF-189(+)] showed almost double the number and density of vessels compared with TSP-2(+)/VEGF-189(–) colon cancers. The prognostic significance of neovascularization in solid neoplasms has been demonstrated in malignant melanoma (Graham et al, 1994), breast (Weidner et al, 1993), prostate (Weidner et al, 1991), bladder (Grossfeld et al, 1997), stomach (Maeda et al, 1995) and non-small-cell lung cancer (Macchiarini et al, 1992). Vessel counts were significantly correlated with time to recurrence in node-negative colon cancer (Takahashi et al, 1997). This study showed that stromal angiogenesis is finely controlled by TSP-2 as well as VEGF-189 expression, and that *TSP-2* expression is of prognostic significance in colon cancer. Angiogenic phenotype which may be able to support tumorigenicity can arise in a stepwise fashion in response to both a decrease in the secretion of inhibitors and the sequential up-regulation of the secretion of inducers of angiogenesis (Volpert et al, 1997). The close correlation and local balance between tissue stable TSP-2 and cell-associated VEGF-189 shown in this study are critical for the progression of colon cancer. *TSP-1* expression showed no significant correlations with angiogenesis in colon cancer.

Oncogenes influence angiogenesis by encoding secreted proteins that are themselves angiogenic factors or by stimulating cells to secrete angiogenic factors as well as enzymes that enhance angiogenesis (Stellmach et al, 1996). There was no apparent correlation between activated *ras* oncogene and *TSP-2* expression, whereas the K-*ras* oncogene is known to stimulate secretion of VEGF (Rak et al, 1995). We also detected *TSP-2* expression in five out of six patients with transforming growth factor β (TGF-β) expression, although TGF-β was reported to be associated with *TSP* gene expression (Laiho et al, 1991). It is not clear which factors affect TSP-2 mRNA expression in colon cancers.

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