Expression of Thrombospondin-derived 4N1K Peptide-containing Proteins in Renal Cell Carcinoma Tissues Is Associated with a Decrease in Tumor Growth and Angiogenesis[1]

Yasuyoshi Miyata, Shigehiko Koga, Kousuke Takehara, Hiroshi Kanetake, and Shigeru Kanda[2]

Department of Urology [Y. M., S. Ko., K. T., H. K., S. Ka.] and Department of Molecular Microbiology and Immunology, Division of Endothelial Cell Biology [S. Ka.], Nagasaki University Graduate School of Medical Science, Nagasaki 852-8501, Japan

ABSTRACT

Purpose: **We have reported that the 4N1K peptide (KRFYVVMWKK) from thrombospondin (TSP) 1 has antiangiogenic activities. The goal of this study was to examine whether the expression of 4N1K-containing proteins correlates with reduced growth of human renal cell carcinoma (RCC).**

Experimental Design: **We examined the expression of 4N1K-containing proteins and TSP1, microvessel density, proliferation index, and apoptotic index in 119 surgically excised RCC tissues by immunohistochemical techniques. The correlation between the above variables and clinicopathological features was analyzed statistically.**

Results: **The antibody raised against the 4N1K peptide recognized protein fragments of matrix metalloproteinase 3-digested TSP1 and positively stained the sections of renal cancer tissues. These reactions disappeared when the antibody was preincubated with immobilized 4N1K peptide, suggesting that the reactions were 4N1K peptide specific. Although TSP1 expression did not correlate with various clinicopathological features and tumor size, all 4N1K-positive tumors were locally confined and of significantly smaller size (median, 3.3 cm; range, 2.0–4.4 cm) than 4N1K-negative tumors (median, 5.2 cm; range, 2.8–8.8 cm; $P < 0.001$). 4N1K-positive tumors exhibited significantly lower microvessel density and higher apoptotic index of tumor cells than 4N1K-negative tumors ($P < 0.001$ and $P = 0.042$, respectively).**

Conclusions: **Our data suggest that expression of 4N1K-containing proteins in tumor tissues is associated with reduced angiogenesis and tumor growth; thus, it would be a potentially predictive marker for progression of RCC.**

INTRODUCTION

TSP1[3] is a multifunctional protein that belongs to a protein family of five members with similar structures (1, 2). TSP1 acts as a positive or negative regulator of angiogenesis, an activator of latent transforming growth factor β1, and a stimulator for tumor cell invasion and metastasis (1–5). These divergent functions of TSP1 may positively or negatively influence the correlation between TSP1 expression and tumor growth as well as angiogenesis in solid tumors (4, 5).

To examine the biological functions of TSP1, synthetic peptides derived from TSP1 have been used. Col I overlap (NGVQYRN) with a peptide from the procollagen homology region has been found to inhibit FGF-2-mediated motility of endothelial cells as well as FGF-2-induced neovascularization in rat cornea (6). The peptide from type I repeats (GSVTGG) inhibits the association of TSP1 with CD36 (7), and the other peptides from type I repeats (KRFK and WSHSPW) are involved in activation of transforming growth factor β1 (8, 9). A peptide from the NH_2-terminal region (ELTGAARKGSGRRLVKGPD) transduces the signal for disassembly of focal adhesions in a phosphatidylinositol 3′-kinase-dependent manner by bovine aortic endothelial cells (10), and ligation of $\alpha_3\beta_1$ integrin by the peptide from the NH_2-terminal region (FQGVLQNVRFVF) regulates endothelial cell proliferation (11).

The 4N1K peptide (KRFYVVMWKK) derived from the COOH-terminal cell binding domain of TSP1 is identified as a ligand for integrin-associated protein (also known as CD47; Ref. 12). Binding of 4N1K to integrin-associated protein modifies particular integrin function, such as that of $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins, resulting in the modulation of integrin-dependent cell spreading and motility (13, 14). These data suggest that 4N1K peptide or proteins containing the 4N1K sequence may enhance the invasion and metastasis of tumor cells. In contrast, we have previously shown that the 4N1K peptide exhibited antiangiogenic activity in both *in vitro* and *in vivo* models (15). Anti-4N1K peptide antibody blocked the inhibitory action of mature TSP1 on tube formation of endothelial cells, suggesting that the 4N1K sequence was responsible for antiangiogenic action of

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[2] To whom requests for reprints should be addressed, at Departments of Urology and Molecular Microbiology and Immunology, Nagasaki University Graduate School of Medical Science, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Phone: 81-95-849-7340; Fax: 81-95-849-7343; E-mail: shigeruk@net.nagasaki-u.ac.jp.

[3] The abbreviations used are: TSP, thrombospondin; FGF, fibroblast growth factor; MVD, microvessel density; MMP, matrix metalloproteinase; RCC, renal cell carcinoma; LI, labeling index; AI, apoptotic index; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PVDF, polyvinylidene difluoride; TNM, tumor-node-metastasis.

TSP1 in the study. In addition, the 4N1K sequence is also present in the COOH-terminal domain of TSP2, which is also known as a potent antiangiogenic factor (16, 17).

To determine whether the 4N1K-containing proteins are involved in suppression of tumor growth, we examined the expression of 4N1K-containing proteins in sections of surgically removed RCC tissues. Our results showed that the expression of 4N1K-containing proteins in such tissues was significantly associated with smaller tumor size and lower MVD. Therefore, it seems likely that evaluation of 4N1K-containing proteins in tumor tissues would be a predictive marker for patients with RCC.

MATERIALS AND METHODS

Patient Characteristics. A total of 119 patients, who underwent surgical excision of RCC at Nagasaki University Hospital between 1989 and 2001, were reviewed. They included 84 men and 35 women, ranging in age from 34 to 70 years (mean age, 57.1 years). All tissue specimens were obtained at surgical resection. Extracapsular invasion, lymph node metastasis, tumor grade, vascular invasion, and tumor size were examined histopathologically. Patients who received any preoperative therapies, such as the embolization of renal artery, were excluded from the study. Chest X-ray photography, computed tomography, and bone scanning were performed for evaluation of distant metastasis. Staging of RCC was performed according to the TNM staging system published in 1997 (18). Among 119 patients, 82 patients had locally confirmed tumors, 10 patients had lymph node metastasis, and 17 patients demonstrated distant metastasis, such as lung and bone.

Antibodies. Rabbit polyclonal antibody raised against the 4N1K peptide was purified with immobilized 4N1K peptide on agarose beads as described previously (15). A monoclonal antibody against human TSP1 (Ab-1) was obtained from Calbiochem-Novabiochem International (Cambridge, MA). Anti-Ki-67 antibody (clone MIB-1), which was used in the examination of proliferation index of tumor cells, was purchased from Dako (Grostrup, Denmark). Anti-CD31 monoclonal antibody (clone 1A10) to visualize endothelial cells was from Novocastra (Newcastle, United Kingdom).

Immunoblotting. Ten μg of human TSP1 (Calbiochem-Novabiochem Corp., San Diego, CA) were either treated with 2.5 microunits of the purified catalytic domain of MMP3 (Calbiochem-Novabiochem Corp.) or left untreated at 37°C for 60 min and electrophoresed in 9.5% polyacrylamide gels, followed by electrotransfer onto PVDF membranes (Millipore, Bedford, MA). Membranes were soaked in 5% nonfat dried milk in PBS for 1 h and incubated with anti-4N1K antibody. The membranes were washed and incubated with peroxidase-conjugated antirabbit IgG. Particular proteins were visualized by the enhanced chemiluminescence reaction. After stripping, the membranes were incubated with anti-4N1K antibody, which had been preincubated with 4N1K peptide-immobilized agarose beads for 30 min at 4°C, and immunoblotting was performed.

Immunohistochemical Analysis. All immunohistochemical studies were performed with formalin-fixed, paraffin-embedded sections. Five-μm sections of tissues were deparaffinized with three changes of xylene and rehydrated sequentially with 80%, 90%, 95%, and 100% ethanol; and then specimens were either heated at 95°C for 40 min for TSP1 or treated with 1 ng/ml trypsin (T-8128; Sigma Chemical Co., St. Louis, MO) at 37°C for 3 min for 4N1K-containing proteins. To examine the proliferation index and MVD, sections that were deparaffinized and dehydrated were treated with 0.01 M sodium citrate buffer (pH 6.0) at 121°C for 15 min. After the antigen retrieval treatment, sections were cooled at room temperature for 60 min. Endogenous peroxidase was blocked by the immersion of sections in 3% hydrogen peroxide solution for 30 min. Nonspecific binding was blocked by incubation with PBS containing 5% skin milk, 2% BSA, and normal goat serum for 60 min, and then the sections were incubated with 4 μg/ml anti-TSP1 antibody, 100 μg/ml anti-4N1K antibody, anti-Ki-67 antibody (diluted 1:100), and anti-CD31 antibody (diluted 1:50) at 4°C overnight, respectively. As negative controls for anti-TSP1 antibody or anti-4N1K antibody, consecutive sections were treated with 4 μg/ml normal mouse IgG alone or with 100 μg/ml preimmune rabbit IgG, respectively. As a negative control in the indicated sections, preincubation of anti-4N1K antibody with 4N1K peptide-immobilized agarose beads was used. As negative controls for anti-Ki-67 antibody and anti-CD31 antibody, sections were incubated with PBS instead of the primary antibody. After the incubation with primary antibodies, sections were treated with DAKO biotin blocking system (Dako Corp., Carpinteria, CA) for inhibition of endogenous biotin. Sections were washed extensively and then incubated with biotinylated antimouse IgG or antirabbit IgG, followed by incubation with horseradish peroxidase-conjugated avidin. Peroxidase was visualized by the use of liquid 3,3′-diaminobenzidine substrate kit (Zymed Laboratories, Inc., San Francisco, CA), and sections were counterstained with hematoxylin.

Detection of Apoptotic Cells. Tissue sections that were deparaffinized and rehydrated were incubated with 20 μg/ml proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) for 15 min at room temperature. After washing with PBS, sections were immersed for 5 min in 3% hydrogen peroxide. Apoptotic cells were detected by Apop Tag *In Situ* Apoptosis Detection Kit (Intergen Company, Purchase, NY), based on TUNEL assay. Sections were counterstained with 2% methyl green. Negative controls consisted of consecutive sections in which the terminal deoxynucleotidyl transferase enzyme was omitted.

Quantitative Analysis. Necrotic areas, those with prominent hyalinization, and hemorrhagic areas were excluded from analysis. The presence of TSP1 or 4N1K immunoreactivity was assessed primarily by the methods described by Grossfeld *et al.* (19). In brief, no or negligible/equivocal immunoreactivity of TSP1 or 4N1K in the tumor area was defined as negative, and moderate or high TSP1 or 4N1K staining in tumor area was defined as positive. The Ki-67 LI was determined as the ratio of positive cells to all tumor cells as a percentage (at least 1000 tumor cells in 3–5 different fields/specimen were calculated under ×400 magnification). The MVD was estimated by counting the number of CD31-positive lumens in tumor area representative of the highest MVD at ×200 magnification. The AI was represented as the number of TUNEL-positive tumor cells/total number of tumor cells, and at least 1000 cells were examined in each section. Evaluation of all immunohistochemical data was performed by one investigator (Y. M.) and subse-

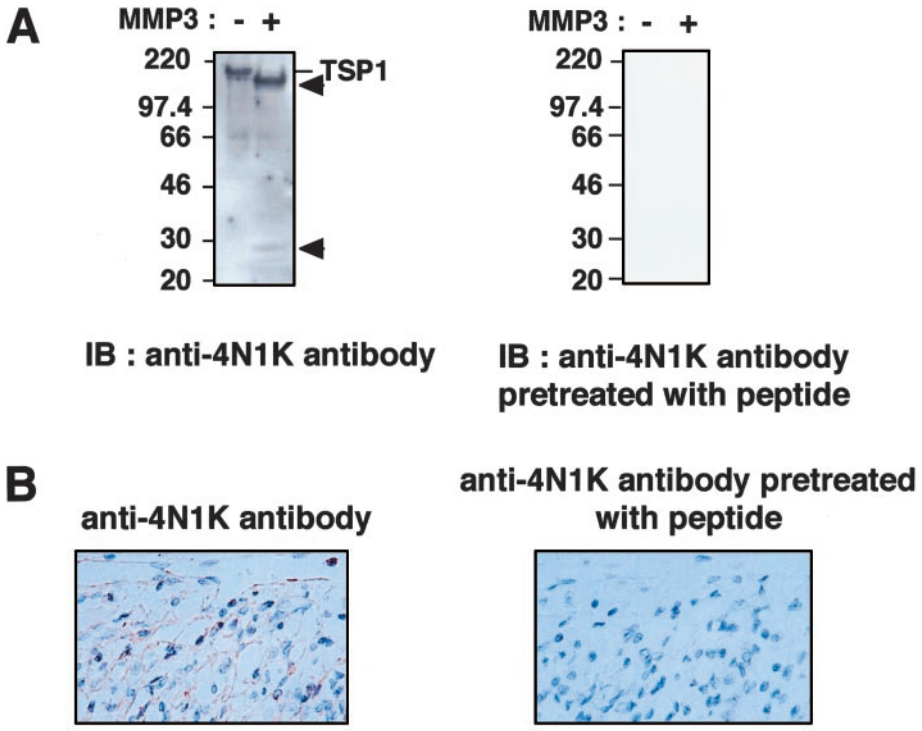

Fig. 1 *A,* immunoblot analysis for characterization of the affinity-purified anti-4N1K peptide antibody. Ten μg of purified TSP-1 were treated with either 2.5 microunits of the purified catalytic domain of MMP3 or left untreated at 37°C for 60 min and electrophoresed, followed by electrotransfer onto PVDF membranes. TSP1 and 4N1K-containing proteins (*arrowheads*) were visualized by anti-4N1K antibody (*left panel*). After stripping of membranes, the anti-4N1K antibody, preincubated with 4N1K peptide-immobilized agarose beads for 30 min at 4°C, was used as the first antibody (*right panel*). *B,* positive staining of 4N1K-containing proteins in tumor tissue disappeared when the antibody was preincubated with 4N1K peptide-immobilized agarose beads. Sequential tissue sections of RCC were deparaffinized, treated with trypsin, and stained with either anti-4N1K antibody or the antibody preincubated with 4N1K peptide-immobilized beads. Reproducible results were obtained from two independent experiments. ×200.

quently reviewed by two other investigators who were blinded to the clinical characteristics at different times (K. T. and S. Ko.).

Statistical Analysis. The normal distribution of the data was initially confirmed for each variable by generating histograms, and normally distributed variables (age, Ki-67 LI, MVD, and AI) were showed as mean ± SD. On the other hand, the variable that did not show normal distribution (tumor size) was expressed as median, with interquartile ranges. Differences in staining patterns between normally distributed variables were examined by the Student's *t* test, and differences in staining patterns for other continuous variables were examined by the Mann-Whitney *t* test. The χ^2 test was used for categorical comparison of the data. To investigate the relationship between TSP1 and the 4N1K immunoreactivity, we used Spearman's rank correlation coefficient. All statistical tests were two sided, and significance was defined as $P < 0.05$. All statistical analyses were performed on a personal computer with the statistical package StatView for Windows (Version 5.0).

RESULTS

Anti-4N1K Antibody Can Recognize 4N1K-containing Fragments of TSP1. We have previously shown that the 4N1K peptide from human TSP1 inhibited FGF-2-induced tube formation of murine brain capillary endothelial cells as well as FGF-2-induced neovascularization in mouse cornea (15). We have also demonstrated that affinity-purified anti-4N1K antibody blocked the inhibitory action of both TSP1 and 4N1K peptide on FGF-2-induced tube formation by murine brain endothelial cells. Human renal carcinoma cells express a panel of proteases, such as MMP3 (20). To examine whether the affinity-purified antibody recognizes TSP1 as well as proteolytic fragments of TSP1, purified human TSP1 and MMP3-treated TSP1 were electrophoresed and then transferred onto PVDF membranes. As shown in Fig. 1*A*, the antibody recognized untreated TSP1 as well as MMP3-digested fragments of TSP1 (M_r 145,000 and M_r 28,000, respectively) by immunoblot analysis. Preincubation of the antibody with 4N1K-conjugated agarose beads abolished the reaction (Fig. 1*A*). These data indicate that the antibody was able to detect both TSP1 and 4N1K-containing proteolytic fragments of TSP1. We then tested whether the antibody could detect 4N1K-containing proteins in formalin-fixed, paraffin-embedded sections of human renal carcinoma tissues. As shown in Fig. 1*B*, the 4N1K antibody reacted with a section of RCC. Interstitial tissue and the plasma membrane of tumor cells were positively stained. The reaction disappeared when the antibody was absorbed with 4N1K peptide-immobilized agarose beads. These data suggest that the antibody could specifically detect 4N1K-containing proteins in human tissue.

Detection of TSP1 and 4N1K-containing Proteins in Renal Carcinoma Tissues. The presence of TSP1 or 4N1K-containing proteins in human renal cancerous tissues was examined by immunohistochemistry. The recognition site of anti-TSP1 antibody, used in the present study, was not identified. In some sections of RCC, TSP1 and 4N1K-containing proteins were positively stained. As shown in Fig. 2, *A* and *C*, TSP1 and 4N1K-containing protein were mainly detected in interstitial tissue of tumor tissue. When preimmune rabbit IgG or nonimmune mouse IgG was used as the first antibody,

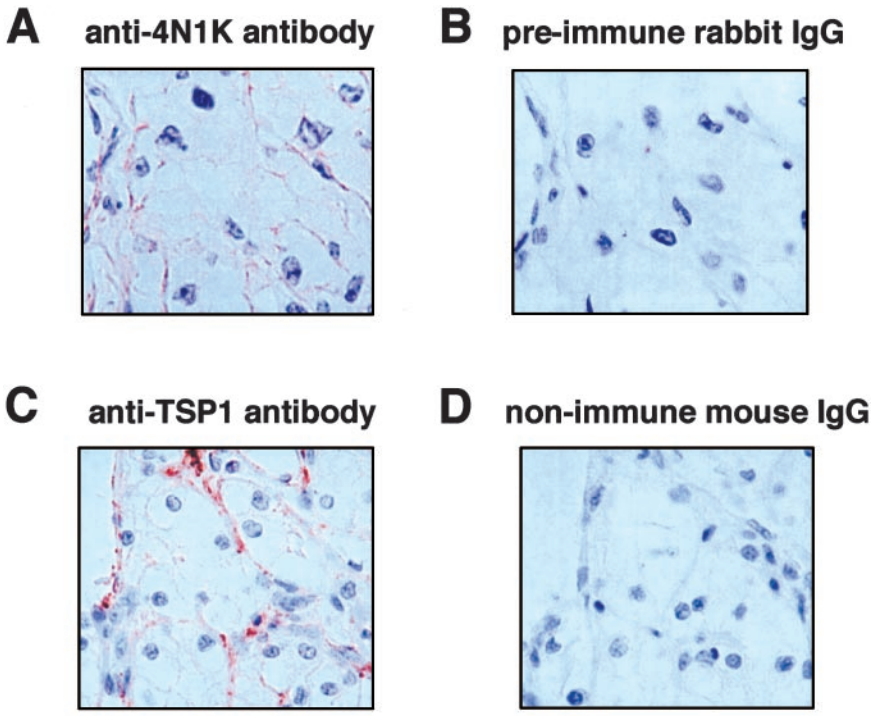

Fig. 2 Detection of TSP1 protein or 4N1K-containing proteins in formalin-fixed, paraffin-embedded tissues by immunohistochemical technique. *A,* positive staining for 4N1K immunoreactivity in tumor tissue. Deparaffinized specimens were treated with 1 ng/ml trypsin at 37°C for 3 min and incubated with rabbit affinity-purified anti-4N1K antibody. *B,* the same tissue sections shown in *A* were incubated with preimmunized rabbit IgG as a negative control. *C,* positive staining for TSP1 immunoreactivity in tumor tissue. Deparaffinized specimens were heated at 95°C for 40 min and incubated with mouse monoclonal anti-TSP1 antibody. *D,* the same tissue sections shown in *C* were incubated with normal mouse IgG as a negative control.

positive staining of sequential tumor tissues was not detected (Fig. 2, *B* and *D*).

Existence of 4N1K-containing Proteins, but not TSP1, Inversely Correlates with Tumor Progression. The expression of 4N1K in RCC tissues did not correlate significantly with that of TSP1 ($P = 0.479$). Of 70 TSP-1-negative tumors, 53 tumors were negative for 4N1K, whereas 17 were positive. In contrast, 34 of 49 TSP1-positive tumors exhibited negative staining with anti-4N1K antibody, and 15 were positively stained. The results suggest that positive staining for 4N1K seems to be independent of that of TSP1. We then compared the relationship between expression of 4N1K-containing protein or TSP1 and clinicopathological features of patients with RCC. As shown in Table 1, positive staining for TSP1 did not correlate significantly with tumor growth and metastasis. However, positive staining for 4N1K correlated inversely with tumor growth, lymph node and distant metastases, and vascular invasion. Thirty of 32 RCCs (93.8%) demonstrated T_1 disease, and all 4N1K-positive tumors were locally confined tumors and negative for lymph node and distant metastases. As shown in Fig. 3, there was a significant correlation between tumor size and positive staining for 4N1K, but not TSP1. The median diameter of 4N1K-negative tumors was 5.2 cm (interquartile range, 2.8–8.8 cm), and that of 4N1K-positive tumors was 3.3 cm (interquartile range, 2.0–4.2 cm), respectively ($P < 0.001$). These data suggest that the 4N1K-containig proteins correlate significantly with inhibition of tumor growth and progression of RCCs.

Expression of 4N1K-containing Proteins Correlates Significantly with Reduced Tumor Angiogenesis. For better understanding of the mechanism underlying the retarded growth of renal carcinomas that contain the 4N1K-containing proteins, we examined the MVD in tumor tissue and the apoptosis and proliferation indices of tumor cells in cancerous tissues of locally confined RCC. As shown in Table 2, the presence of either TSP1 or 4N1K did not correlate with the proliferation index of tumor cells, which was assessed by Ki-67 staining of nuclei. However, MVD, examined by staining of endothelial cells with anti-CD31 antibody, was inversely and significantly correlated with the expression of 4N1K-containing proteins. In addition, the AI of tumor cells was markedly higher in the 4N1K-positive group than in the 4N1K-negative group. Expression of TSP1 did not correlate with MVD and AI of tumor cells. These data suggest that expression of 4N1K-containing proteins seems to be associated with reduced tumor angiogenesis, resulting in induction of apoptosis of tumor cells.

DISCUSSION

In the present study, we show that expression of 4N1K-containing proteins in RCC is inversely associated with tumor size and MVD. In contrast, immunohistochemical detection of TSP1 did not correlate with tumor features and angiogenesis. Among 32 tumors positive for 4N1K, 30 (93.8%) were of T_1 disease, and no tumor exhibited T_3 or T_4 or metastatic disease. In locally confined tumors, the 4N1K-positive tumors demonstrated significantly lower MVD than the 4N1K-negative tumors. In contrast, positivity for TSP1 did not correlate with tumor size or MVD. Thus, our results suggest that 4N1K-containing proteins seem to inhibit angiogenesis, resulting in retarded growth of RCCs.

Table 1 The relationship between TSP1 expression or 4N1K immunoreactivity and clinicopathological features of RCC

T, N, and M classification were assessed according to the 1997 TNM grading system. Vascular invasion was examined microscopically with the removed tissues.

	No. of patients (%)	TSP1 expression (%)		4N1K immunoreactivity (%)	
		Negative (n = 70)	Positive (n = 49)	Negative (n = 87)	Positive (n = 32)
T classification					
T_1	69 (58.0)	41 (58.6)	28 (57.1)	39 (44.8)	30 (93.8)
T_2	17 (14.3)	8 (11.4)	9 (18.4)	15 (17.2)	2 (6.2)
T_3	28 (23.5)	19 (27.1)	9 (18.4)	28 (32.2)	0 (0.0)
T_4	5 (4.2)	2 (2.9)	3 (6.1)	5 (5.7)	0 (0.0)
P		0.448		<0.001	
N classification					
N_0	109 (91.6)	63 (90.0)	46 (93.9)	77 (88.5)	32 (100.0)
N_1 or N_2	10 (8.4)	7 (10.0)	3 (4.1)	10 (11.5)	0 (0.0)
P		0.453		0.045	
M classification					
M_0	102 (85.7)	62 (88.6)	40 (81.6)	70 (80.5)	32 (100.0)
M_1	17 (14.3)	8 (11.4)	9 (18.4)	17 (19.5)	0 (0.0)
P		0.287		0.007	
Vascular invasion					
Negative	89 (74.8)	50 (71.4)	39 (79.6)	60 (69.0)	29 (90.6)
Positive	20 (16.8)	20 (29.6)	10 (20.6)	27 (31.0)	3 (9.4)
P		0.313		0.016	

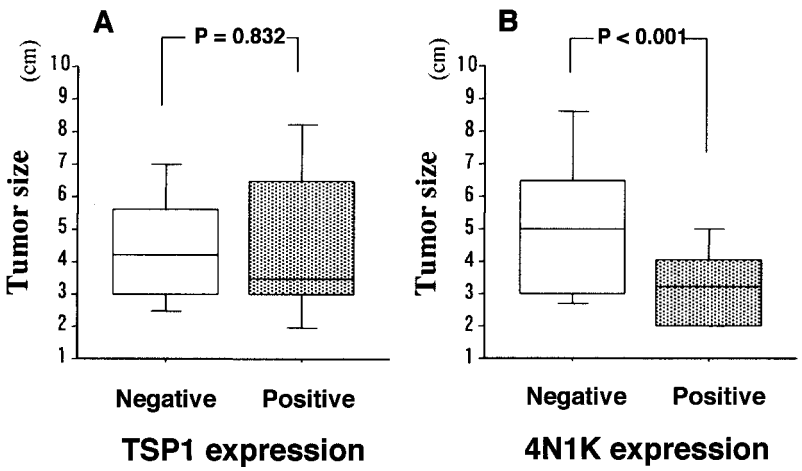

Fig. 3 Relationship between the largest diameter of each tumor and immunohistochemical staining for TSP1 or 4N1K-containing proteins. *A*, there was no significant correlation between the mean largest diameter of tumors and TSP1 expression. *B*, the largest tumor diameter in the 4N1K-positive group was significantly smaller than that in the 4N1K-negative group. *Bars* represent the mean largest diameter (cm) of tumor ± 95% confidence interval.

Sections of tumor tissues with positive TSP1 staining did not match well with 4N1K-positive tumors. Of 49 tumors positive for TSP1, 15 were 4N1K positive, and 34 were 4N1K negative. Conversely, of 70 TSP1-negative tumors, 53 were 4N1K-negative tumors, and 17 were 4N1K-positive tumors. Renal carcinoma cells produce a number of proteases, such as MMP3, to allow them to invade the surrounding interstitial tissues. The fact that the carcinoma tissues were positively stained by the anti-4N1K antibody indicates the presence of proteolytic peptide fragments that contain the 4N1K sequence, KRFYVVMWKK. The sections positive for TSP1 and negative for 4N1K may lack the 4N1K-containing fragments in expressed TSP1 proteins. Alternatively, 4N1K-containing proteins in RCC tissues may partly originate from TSP2, which is also a negative regulator of angiogenesis.

Previous studies have suggested that the correlation between TSP1 expression and tumor angiogenesis seems to depends on the type of tumor. TSP1 expression correlates inversely with the MVD in malignant tumors of the prostate and colon (21, 22). However, no correlation was observed in various other carcinomas (23–25), and a positive relationship was reported in pancreatic cancer (26). Because TSP1 plays various roles in angiogenesis, detection of TSP1 may not be useful for evaluating its roles in angiogenesis. At present, there is no test designed to detect TSP1 proteins using a sequence-specific antibody. It has been shown that two in-

Table 2 Ki-67 LI, MVD, and AI of tumor cells in locally confined RCC

To examine the proliferation or apoptosis of tumor cells, sections of tumor tissues were stained with anti-Ki-67 antibody or by the TUNEL method. Ki-67 LI and AI were expressed as positively stained nuclei/total nuclei × 100 (%). For examination of MVD, sections were stained with anti-CD31 antibody to visualize endothelial cells, and MVD was expressed as the number of CD31-positive microvessels/mm^2 in tumor tissues. Data represent the mean values ± 95% confidence interval.

	TSP1 expression			4N1K immunoreactivity		
	Negative (n = 48)	Positive (n = 34)	P	Negative (n = 50)	Positive (n = 32)	P
Ki-67 LI (%)	5.3 ± 4.9	4.7 ± 3.8	0.249	5.2 ± 4.7	4.8 ± 4.1	0.707
MVD (mm^2)	130 ± 55	121 ± 44	0.436	141 ± 51	104 ± 41	<0.001
AI (%)	1.76 ± 0.79	1.85 ± 2.03	0.770	1.51 ± 0.62	2.24 ± 2.10	0.023

dependent regions within the second and third type I repeats exerted antiangiogenic activities by distinct mechanisms, suggesting that the use of domain-specific antibodies might still be insufficient to examine the roles of TSP1 in *in vivo* angiogenesis.

In the present study, expression of 4N1K-containing proteins did not correlate with the proliferation index of tumor cells. The 4N1K peptide did not affect the proliferation of cultured human renal carcinoma ACHN and VMRC-RCW cells.[4] Therefore, the 4N1K peptide or 4N1K-containing proteins do not seem to have a direct effect on proliferation of renal carcinoma cells.

In conclusion, our data suggested that assays using sequence-specific antibodies, such as anti-4N1K antibody, to detect proteins from TSP are useful tools for the prediction of tumor progression, angiogenesis, and clinical courses of human cancers.

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