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The Pathological Significance and Prognostic Roles of Thrombospondin-1, and -2, and 4N1K-peptide in Bladder Cancer

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Abstract. *Background/Aim: Thrombospondins (TSPs) play a role as inhibitors of angiogenesis under various pathological conditions. The aim of the study was to evaluate the pathological significance and prognostic role of the 4N1K-peptide (KRFYVVMWKK), which is derived from TSP-1 and -2, in bladder cancer. Materials and Methods: Two-hundred and six bladder cancer tissues were examined for expression of TSP-1, TSP-2, and 4N1K-peptide by immunohistochemistry. Cancer cell proliferation, apoptosis, angiogenesis and matrix metalloproteinase (MMP)-9 immunoreactivity were also examined. Results: Expression of TSP-2 and 4N1K-peptide was negatively associated with T stage, metastasis, and grade. TSP-2 expression was negatively associated with cancer cell proliferation and MMP-9 expression, whereas 4N1K-peptide was significantly associated with apoptosis, angiogenesis, and MMP-9 expression. Multivariate analysis showed that 4N1K-peptide expression was a significant predictor of metastasis (hazard ratio=3.90, p=0.002). Conclusion: TSP-2 and 4N1K peptide played important roles in malignant aggressiveness and progression of bladder cancer via complex mechanisms involving cell proliferation, apoptosis, angiogenesis, and MMP-9.*

Thrombospondin (TSP) is a family of glycoproteins composed of 5 subtypes encoded by specific genes (*TSP-1* to *-5*). Many studies have verified that TSP-1 has anti-angiogenic activities under various pathological conditions, including cancers (1-7). However, TSP-1 has also been shown to stimulate malignant aggressiveness of several cancers (8-11). As such, it is still uncertain whether TSP-1 has pro- or anti-cancer effects. *TSP-1* and *TSP-2* genes show a high degree of sequence homology (12). Interestingly, TSP-2 has stronger inhibitory effects on tumor growth and angiogenesis compared to TSP-1 (13, 14). Actually, several investigators have reported that TSP-2 is a negative regulator of cancer-related angiogenesis and tumour aggressiveness in a variety of cancers (4, 15-18). However, other investigators have suggested that TSP-2 is not involved in tumor growth and cancer cell invasion (19, 20). In urothelial cancer, TSP-2 has been reported to be positively associated with pathological features and vascular invasion, and was identified as predictor of worse prognosis for metastasis-free and disease specific survival (21). However, unfortunately, there is no additional study regarding the pathological significance of TSP-2 in bladder cancer (BC), and the detailed molecular mechanisms of such TSP-2-mediated activities in BC tissues are not fully understood.

The biological activities of TSPs are closely related to their derived fragments and proteins (22-24). For example, the collagen I overlap (NGVQYRN) derived from the procollagen homology domain is associated with angiogenesis *in vivo* (22). Therefore, in the present study, we focused on the pathological significance and prognostic roles of TSP-1, -2, and the 4N1K peptide (KRFYVVMWKK), which is derived from the C-terminal cell-binding domain of TSP-1 and TSP-2, for the following reasons: 1) there is no information on the pathological significance and prognostic role of 4N1K-peptide in BC patients despite the fact that it has been suggested as a potential therapeutic target and a useful predictive factor in other cancers (25-27), 2) the pathological molecular mechanisms involved with the TSPs and the 4N1K-peptide are not clear, and 3) comprehensive understanding of the functions/effects of TSPs and their derived peptide is important regarding the treatment and observation strategies in patients with BC.

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Key Words: Thrombospondins, 4N1K-peptide, bladder cancer, pathological features, prognosis.

Materials and Methods

Patients. Two-hundred and six consecutive patients who were diagnosed with urothelial cancer of the urinary bladder were reviewed retrospectively. This study included 162 men and 44 women, ranging in age from 39 to 93 years (median age: 71 years). T stage was divided into the following groups: low- (pTa+1) and high-stage (≥T2). In addition, lymph node metastasis and distant metastasis coalesced into the term metastasis, for statistical analysis. We also examined 20 tissue samples of normal urinary bladders obtained from apparently normal areas of the bladder of patients with transitional cell carcinoma of the upper urinary tract. Survival analyses were performed in patients who had undergone potential curative transurethral resection (n=185). In short, patients with non-metastatic tumor with Ta – 1 and pT2 were enrolled. Among these patients, intravesical therapy was performed for 162 patients (87.6%) as adjuvant therapy. The median duration of follow-up was 57 months (interquartile range=24-95 months).

Immunohistochemistry. Five-micrometer-thick sections were deparaffinized in xylene and rehydrated in ethanol. All sections were subjected to antigen retrieval and then immersed in hydrogen peroxide to block endogenous peroxidase activity. The specificity of the anti-4N1K-peptide has been confirmed in several other reports (25, 26). The other antibodies were obtained as follows: anti-TSP-1 and -TSP-2 from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), anti-Ki-67 and anti-CD34 from Dako Corp. (Grostrup, Denmark), anti-matrix metalloproteinase-9 (MMP-9) from Daiichi Fine Chemical (Toyama, Japan), and anti-cleaved caspase-3 antibody from R & D Systems, Inc. (Abingdon, UK). Sections were incubated with the primary antibody at 4˚C overnight. Then, the sections were washed extensively and treated with peroxidase using the labeled polymer method with DAKO EnVision+TM Peroxidase (Dako Corp., Carpinteria, CA, USA). The peroxidase reaction was visualized with the liquid DAB substrate kit (Zymed Laboratories Inc., San Francisco, CA, USA). Sections were counterstained with hematoxylin. Immunohistochemical staining of a positive control was performed as has been described previously for all antibodies except TSP-2 (28-30). For TSP-2, the spleen was used as a positive control according to the manufacturer's data sheet. For evaluation of immunohistochemical staining for TSP-1, TSP-2, and 4N1K-peptide, the intensity was graded as none, weak, moderate, or strong, and then specimens with moderate or intense staining in >10% of cells were judged as positive. The evaluation of immunohistochemical staining for Ki-67, CD34, and MMP-9 and apoptotic cells were performed as has been previously described (28-30). For all variables, values above the median were considered as the high expression group, and those with staining equal to or less than the median value were considered as the low expression group, for statistical analyses. Slides were examined using an E-400 microscope (Nikon, Tokyo, Japan) producing digital images, which were examined using a computer-aided image analysis system (Win ROOF version 5.0; MITANI, Fukui, Japan). Slides were evaluated twice at different times by three investigators (Y.M., T.M., and A.A.), who were blinded to the clinicopathological features and survival data.

Ethics and statistical analyses. The study was conducted according to the Helsinki II Declaration and it was approved by the Ethics Review Committee of the Nagasaki University Hospital (No. 12052899). Written informed consent was obtained from all the patients involved in our study before their enrollment. Data are expressed as medians (interquartile range), unless otherwise stated. The Mann-Whitney *U*-test was performed for continuous variables, and the chi-square test was used for categorical comparison of the data. The raw and adjusted effects on stage and grade, as well as other pathological factors, were estimated by logistic regression analysis, and are described as odds ratios (OR) with 95% confidence intervals (95%CI), together with the associated *p*-values. The metastasis-free survival rates were compared with Kaplan–Meier analysis and a log-rank test. All statistical analyses were performed using the statistical package StatView for Windows (Version 5.0).

Results

TSP-1, TSP-2, and 4N1K-peptide expression and clinicopathological features. In normal urothelial cells, all specimens were judged as positive for TSP-1, -2, and 4N1K-peptide, because almost all cells showed moderate or strong staining (Figure 1A-C). In cancer tissues, all immunostainings were detected in both the cancer cell cytoplasm and stromal cells (Figure 1D-F). Finally, positively stained ratio of TSP-1, TSP-2, and 4N1K-peptide was 41.7%, 42.7%, and 60.7%, respectively. Chi-square test showed that TSP-1 expression was not significantly associated with TSP-2 (p=0.176) or 4N1K-peptide expression (p=0.093). However, TSP-2 expression was positively associated with 4N1K-peptide expression (p<0.001). As shown in Table I, TSP-1 expression was not significantly related to any pathological feature. However, TSP-2 staining was significantly lower in specimens from patients diagnosed at high T stage, with presence of metastasis, and a high grade than in those from patients diagnosed at low T stage, with absence of metastasis, and a low grade (p<0.001, 0.021, and 0.018, respectively). Similarly, negative relationships were found between 4N1K-peptide expression and T stage, metastasis, and grade (p<0.0.01, 0.025, 0.002, respectively). On the other hand, a multivariate analysis model including all pathological features and expression of TSP-1 and -2 demonstrated that 4N1K-peptide expression was independently associated with TSP-2 expression (OR=3.00, 95%CI=1.58-5.72, p<0.001), but not with TSP-1 expression (Table II).

Correlations with cancer-related factors. Relationships between the expression of TSP-1, TSP-2, and 4N1K-peptide and proliferation index (PI), apoptotic index (AI), microvessel density (MVD), and MMP-9 expression are shown in Table III. With regard to cancer cell proliferation, a negative correlation was detected with TSP-2 expression (p=0.005), but not with TSP-1 and 4N1K-peptide expression. By contrast, AI and MVD were significantly associated with 4N1K-peptide expression (p<0.001 and 0.024, respectively), but not with the expression of TSP-1 or TSP-2. The AI in the TSP-2-positive tissues tended to be higher than that in TSP-

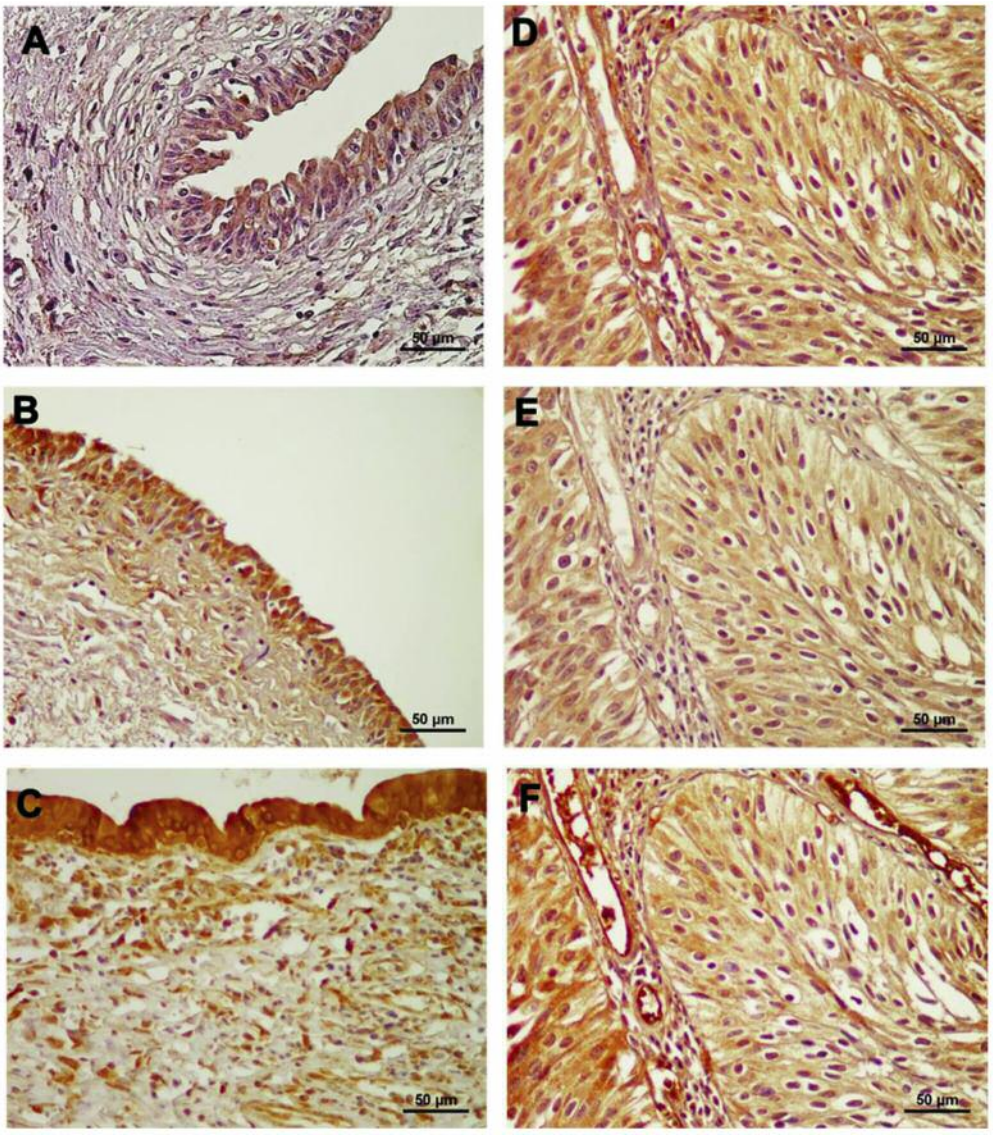

Figure 1. *Representative examples of TSP-1, TSP-2, and 4N1K-peptide expression. Immunohistochemistry for TSP-1, TSP-2, and 4N1K-peptide detected in normal urothelial cells (A-C) and cancer cells (D-F). In normal urothelium, moderate or strong staining was detected in almost all cells. There was no remarkable difference in staining patterns of the cells in cancer tissues. Although vascular endothelial cells (arrows) were mainly stained for 4N1K-peptide (F), such staining was not found for TSP-2 (E).*

2-negative tissues; however, the difference did not reach a statistical significance (p=0.071). MMP-9 expression was negatively associated with the expression of TSP-2 (p<0.001) and 4N1K-peptide (p=0.005). Furthermore, multivariate analysis model including all pathological features showed that TSP-2 expression was significantly associated with MMP-9 expression (OR=0.33, 95%CI=0.17-0.64, p=0.001), and a similar independent correlation was detected between 4N1K-peptide expression and MVD (OR=0.52, 95%CI=0.29-0.95, p=0.033; Table IV).

Survival analyses. As shown in Figure 2A, there was no significant difference in the occurrence of subsequent metastasis according to TSP-1 expression. However, the metastasis-free survival periods in patients with TSP-2-positive tissues were significantly longer than those with negative TSP-2 expression (p<0.001, Figure 2B). A similar finding was obtained with respect to 4N1K-peptide expression (p<0.001, Figure 2C). In this study population, the subsequent metastasis-free survival period was significantly associated with a high T stage (HR=7.48, 95%CI=3.76-14.90, p<0.001)

Table I. *Correlation with clinicopathological features.*

	Thrombosponin-1		Thrombospondin-2		4N1K-peptide	
	Negative (n=120)	Positive (n=86)	Negative (n=118)	Positive (n=88)	Negative (n=81)	Positive (n=125)
Age (years)						
≤71	61 (50.8)	43 (50.0)	55 (46.7)	49 (55.7)	40 (49.4)	64 (51.2)
>71	59 (49.2)	43 (50.0)	63 (53.3)	39 (44.3)	41 (50.6)	61 (48.8)
p-Value	0.906		0.198		0.799	
Gender						
Male	94 (78.3)	68 (791.)	91 (77.1)	71 (80.7)	62 (76.5)	100 (80.0)
Female	26 (21.7)	18 (20.9)	27 (22.9)	17 (19.3)	19 (23.5)	25 (20.0)
p-Value	0.899		0.537		0.554	
T stage						
Ta	37 (30.8)	23 (26.7)	26 (22.0)	34 (38.6)	13 (16.0)	47 (37.6)
T1	50 (41.7)	40 (46.5)	49 (41.5)	41 (46.6)	35 (43.2)	55 (44.0)
T2	18 (15.0)	10 (11.6)	21 (17.8)	7 (8.0)	12 (14.8)	16 (12.8)
T3	8 (6.7)	10 (11.6)	12 (10.2)	6 (6.8)	12 (14.8)	6 (4.8)
T4	7 (5.8)	3 (3.5)	10 (8.5)	0 (0.0)	9 (11.1)	1 (0.8)
p-Value	0.566		0.002		<0.001	
Low (Ta +1)	87 (72.5)	63 (73.3)	75 (63.6)	75 (85.2)	48 (59.3)	102 (81.6)
High (T2 – 4)	33 (27.5)	23 (26.7)	43 (36.4)	13 (14.8)	33 (40.7)	23 (18.4)
p-Value	0.904		<0.001		<0.001	
Metastasis						
Absence	108 (90.0)	77 (89.5)	101 (85.6)	84 (95.5)	68 (84.0)	117 (93.6)
Presence	12 (20.0)	9 (10.5)	17 (14.4)	4 (4.5)	13 (16.0)	8 (6.4)
p-Value	0.913		0.021		0.025	
Grade						
Low	44 (36.7)	42 (48.8)	41 (34.7)	45 (51.1)	23 (28.4)	63 (50.4)
High	76 (63.3)	44 (51.2)	77 (65.3)	43 (48.9)	58 (71.6)	62 (49.6)
p-Value	0.081		0.018		0.002	
Adjuvant Tx						
Nothing	25 (20.8)	19 (22.1)	27 (22.9)	17 (19.3)	16 (19.8)	28 (22.4)
Performed	95 (79.2)	67 (77.9)	91 (77.1)	71 (80.7)	65 (80.2)	97 (77.6)
p-Value	0.828		0.537		0.651	

Data are shown as number (%) of patients. Tx: Therapy.

and high grade (HR=7.47, 95%CI=2.90-19.25, $p<0.001$) according to the univariate analyses. Therefore, the independent roles of the expression of TSP-2 or 4N1K-peptide were analyzed using multivariate analysis models including pathological features (Table V). In Model A, including T stage, grade, and TSP-2 or 4N1K-peptide expression, TSP-2 and 4N1K-peptide expression were identified as independent factors to predict the occurrence of subsequent metastasis (Table V). However, in model B, which included all risk factors, 4N1K-peptide expression was also identified as a significant predictor (HR=3.90, 95%CI=1.68-9.07, $p=0.002$), whereas TSP-2 expression was not (Table V).

Table II. *Multivariate analysis of the correlation between 4N1K-peptide expression and clinicopathological features and expression of TSP-1 and -2.*

	For 4N1K-peptide expression		
	Odds ratio	95%CI	*p*-Value
T stage: low	1.90	0.90-4.04	0.095
Metastasis: absent	1.36	0.44-4.16	0.596
Grade: low	1.58	0.82-3.02	0.172
TSP-1: positive	1.83	0.98-3.44	0.059
TSP-2: positive	3.00	1.58-5.72	<0.001

TSP: Thrombospondin; CI: confidence interval.

Discussion

Our results demonstrated that the expression of TSP-2 and 4N1K-peptide are negatively related with the malignant potential, tumor progression, and prognosis of patients with BC. However, TSP-1 does not appear to play a role or has only a minimal or indirect role in these cancer-related parameters. This finding is in stark contrast to several

Table III. *Correlation between malignant aggressiveness and TSP-1, -2, or 4N1K-peptide*

	Thrombospindin-1		Thrombospndin-2		4N1K-peptide	
	Negative	Positive	Negative	Positive	Negative	Positive
PI, %	23.9/8.6	24.1/8.5	24.5/8.3	22.1/8.5	24.9/8.2	23.4/8.7
p-Value	0.878		0.005		0.203	
AI, %	4.35/1.86	4.26/1.89	4.11/1.89	4.58/1.82	3.75/1.83	4.67/1.81
p-Value	0.729		0.071		<0.001	
MVD, /mm^2	62.4/20.5	61.8/18.1	63.0/19.8	61.3/19.9	66.1/21.2	59.8/18.5
p-Value	0.845		0.565		0.024	
MMP-9	55 (45.8)	37 (43.0)	69 (58.5)	23 (26.1)	46 (56.8)	46 (36.8)
p-Value	0.689		<0.001		0.005	

Data are shown as mean/SD or number (%) of positively stained specimens. PI: Proliferation index; AI: apoptotic index; MVD: microvessel density; MMP: matrix metalloproteinase.

Table IV. *Multi-variate analysis of correlation between cancer-related factors and TSP-2 or 4N1K peptide*.*

	Thrombospondin-2			4N1K-peptide		
	OR	95%CI	*p*-Value	OR	95%CI	*p*-Value
Proliferation index						
Over median	0.95	0.52-1.74	0.857	–	–	–
Apoptotic index						
Median or less	–	–	–	0.56	0.31-1.04	0.068
Microvessel density						
Over median	–	–	–	0.52	0.29-0.95	0.033
MMP-9 expression						
Positive	0.33	0.17-0.64	0.001	0.67	0.36-1.29	0.234

OR: Odds ratio; CI: confidence interval; MMP: matrix metalloproteinase. *Adjusted by T stage, metastasis, and grade.

previous studies showing that decreased expression of TSP-1 was related to high malignant potential and poor survival in BC patients (2, 3, 5, 7). In addition, regarding TSP-2, a previous report has shown that its expression was positively associated with malignant aggressiveness and a worse prognosis in BC patients (21). Although we cannot explain the reason for this apparent discrepancy in the pathological roles of TSP-1 and -2, we hypothesize that the differences among studies, including patients' backgrounds, antibodies used, or evaluation methods, might have contributed to these conflicting results. However, TSP-2 has been reported to act as an inhibitor of tumor invasion and metastasis both *in vivo* and *in vitro* in various types of cancers (15, 16, 18). In addition, a recent study has demonstrated that TSP-2 inhibited vascular endothelial growth factor-induced cell proliferation and migration of tumor-associated blood vascular endothelial cells isolated from human bladder cancer (31). These results support our results that TSP-2 expression may play a role as tumor-suppressor and better predictor for prognosis in patients with BC.

In the present study, we demonstrated, for the first time, the pathological significance and roles of 4N1K-peptide in BC. However, similar findings have been reported in other cancers (26, 27), thus, our results add to the growing body of evidence that 4N1K-peptide has anti-cancer effects. According to the results of our multivariate analysis models, we hypothesized that 4N1K-peptide was mainly derived from TSP-2, and not from TSP-1, in BC tissues. Although the mechanism contributing to this difference was not determined, it is possible that TSP-2 predominates and plays important roles in the malignant aggressiveness of BC cells, and consequently, its derived peptide 4N1K also exhibits pathological activities. Our conclusion is based on two key findings: 1) 4N1K-peptide is derived from the C-terminal cell-binding domain, which exists in TSP-2, and 2) TSP-2 and 4N1K-peptide showed similar associations with cancer cell invasion, metastasis, grade, and prognosis, indicating similar pathological roles.

We also observed differences in the pathological roles between TSP-2 and 4N1K-peptide at the molecular level. In

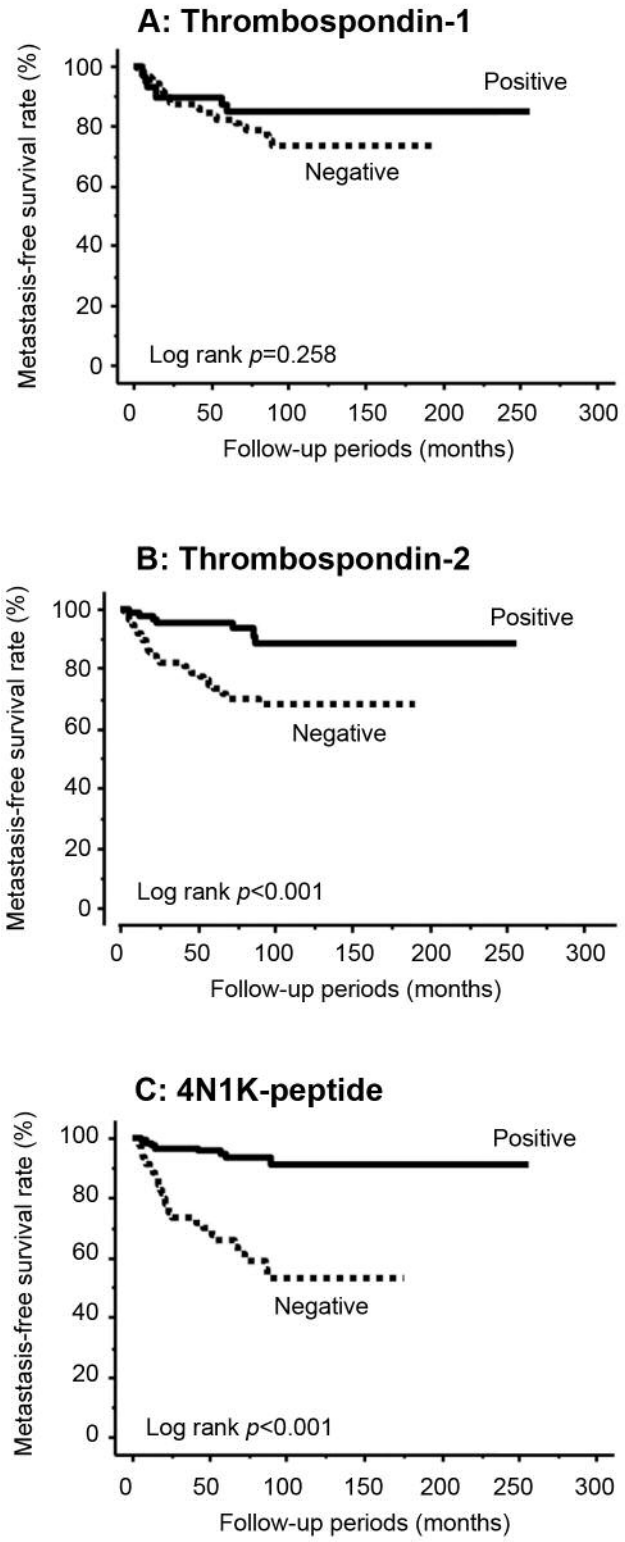

Figure 2. *Kaplan–Meier survival curves for metastasis-free survival. Survival analyses for metastasis-free survival of TSP-1 (A), TSP-2 (B), and 4N1K-peptide expression (C) in bladder cancer patients. Expression of TSP-2 and 4N1K-peptide were significant predictors for metastasis-free survival (p<0.001; B and C) whereas TSP-1 expression was not (A).*

Table V. *Multivariate analyses of the correlation of TSP-1, -2, and 4N1K-peptide and subsequent metastasis.*

	For subsequent metastasis		
	HR	95%CI	*p*-Value
Model A			
T stage: High	4.27	2.10-8.67	<0.001
Grade: High	5.46	2.06-14.46	<0.001
TSP-2: Negative	3.44	1.49-7.96	0.004
T stage: High	4.00	1.94-8.24	<0.001
Grade: High	3.93	1.47-10.54	0.007
4N1K-peptide: Negative	4.92	2.21-10.95	<0.001
Model B			
T stage: High	3.34	1.59-7.01	0.001
Grade: High	4.26	1.59-11.44	0.004
TSP-1: Negative	1.11	0.56-2.19	0.774
TSP-2: Negative	2.26	0.93-5.52	0.073
4N1K-peptide: Negative	3.90	1.68-9.07	0.002

HR: Hazard ratio; CI: confidence interval; TSP: thrombospondin.

short, our multivariate analyses showed that TSP-2 expression was negatively associated with MMP-9 expression, whereas 4N1K-peptide expression was more strongly correlated with angiogenesis in patients with BC. With respect to the relationship between TSP-2 and MMP-9, a similar result has been previously reported in pancreatic cancer cells and in colon cancer (15, 32). Thus, our results extend this relationship to explaining the pathology of BC as well. By contrast, 4N1K-peptide expression was negatively associated with angiogenesis in BC tissues. 4N1K-peptide has been reported to have an anti-angiogenic function *in vivo* (25). In addition, its expression has been shown to be negatively associated with MVD in several cancer tissues (26, 27). These results, suggest that 4N1K-peptide likely plays an anti-angiogenic role in patients with bladder cancer. In fact, our pathological finding showed that strong expression of 4N1K-peptide was detected in microvessels within the tumor area.

In the survival analyses, TSP-2 and 4N1K-peptide were identified as major predictors of subsequent metastasis after operation in patients with BC. As mentioned above, TSP-2 and 4N1K-peptide expression were negatively associated with MMP-9 expression and MVD, respectively. Thus, the prognostic values of TSP-2 and 4N1K-peptide can be adequately explained by these relationships. The multivariate analysis model that included all of the pathological parameters along with TSP-1, TSP-2, and 4N1K-peptide expression showed that only 4N1K-peptide was an independent and significant predictive factor for prognosis. One potential reason for this result might be the dual biological function of TSP-2, with both pro- and anti-cancer effects. This is possible

since TSP-2 is a large multi-domain protein and its pathological activities are regulated by various types of proteins and many molecular pathways that also depend on the tumour microenvironment (33). In short, some domains and derived peptides from TSP-2 may have a pro-angiogenic effect in BC tissues, whereas others might have anti-cancer effects.

One of the limitations of this study was that the results were not confirmed in an *in vitro* experiment. Therefore, more detailed studies are necessary to verify the hypotheses put forth herein. However, the *in vivo* data are important to understand the pathological significance of TSPs and 4N1K-peptide. Furthermore, such detailed investigation has definitely merit in order to develop improved treatment strategies and markers for patients with BC.

Conclusion

TSP-2 and 4N1K-peptide expression were negatively associated with pathological features in patients with BC. Multivariate analysis further showed that 4N1K-peptide was mainly derived from TSP-2. On the other hand, although TSP-2 expression was significantly associated with MMP-9 expression, 4N1K-petide expression was associated with MVD. Multivariate analyses also showed that only 4N1K-peptide expression emerged as a significant predictor for subsequent metastasis. Thus, the pathological roles of 4N1K-peptide are different from those of TSP-2, and 4N1K-peptide would be a more useful predictive marker and potential therapeutic target compared to TSP-1 and TSP-2 in patients with BC.

Conflicts of Interest

None of the Authors has any conflict of interest regarding this study.

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Authors' Contributions

Study concept: YM. Study deign: YM. Clinical data collection: TM, YN, and KO. Immunohistochemical analyses: YM, AA, and KM. Statistical analyses: YM and TM. Manuscript preparation: YN, YM, and KA. Manuscript editing: KT and KO. Manuscript review: HS. All Authors read and approved the final manuscript.

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