Tissue factor expression is associated with recurrence in patients with non-metastatic colorectal cancer

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Purpose: Previous studies have addressed the role of the hypercoagulable state in the pathogenesis of cancer progression and metastasis. In this study, we investigated the association between coagulation factors, including tissue factor (TF) expression, platelet count, and fibrinogen level, and disease recurrence in patients with non-metastatic colorectal cancer.

Methods: Patients who underwent curative resection for stage II or III colorectal cancer between 2000 and 2007 were included in this study. Data from a prospectively maintained database were retrospectively reviewed. TF expression was determined by immunohistochemistry using an anti-TF monoclonal antibody. The Kaplan-Meier method was used to estimate 5-year disease-free survival.

Results: TF was highly expressed in 257 of 297 patients (86.5%). TF expression was not significantly associated with the platelet counts ($P=0.180$) or fibrinogen level ($P=0.281$). The 5-year disease-free survival rate was lower in patients with high TF expression than in patients with low TF expression (72.3% vs. 83.9%, $P=0.074$). In Cox hazard analysis, high TF expression was an independent risk factor for tumor recurrence (hazard ratio [HR] 2.446; 95% confidence interval [CI], 1.054–5.674; $P=0.037$). Undifferentiated histologic type (HR, 2.911; 95% CI, 1.308–6.481; $P=0.009$), venous invasion (HR, 2.784; 95% CI, 1.431–5.417; $P=0.003$), and lymph node metastasis (HR, 2.497; 95% CI, 1.499–4.158; $P<0.001$), were also significantly associated with disease recurrence.

Conclusion: TF expression is associated with a recurrence in patients with non-metastatic colorectal cancer. However, further studies are required to clarify the underlying mechanisms relating TF expression with oncologic outcomes and its potential role as a therapeutic target.

Keywords: Colonic neoplasms, Prognosis, Survival, Immunohistochemistry

INTRODUCTION

Malignancy is a known pro-coagulant state, predisposing to thrombosis. A number of coagulation abnormalities have been reported to be associated with tumor growth, metastasis, and recurrence in different types of solid tumor including esophageal, gastric, pancreatic, ovarian, lung, and colorectal cancer [1-4].

Tissue factor (TF) is a transmembrane glycoprotein that has a cen-

tral role in initiating the coagulation cascade [5,6]. TF expression has been identified immunohistochemically in epithelial tissues, specifically the skin, the mucosal surfaces of the gut, and genitourinary system [7]. However, it has also been found to be overexpressed in cancer cells. A direct association between elevated TF expression and advanced stages of malignancy has been confirmed in several different types of cancers [8-11]. More recent studies have suggested that TF expression contributed to metastasis by interfering with cell signaling pathways and presenting the proteolytic activity by the TF/factor VI activated complex. Therefore, increased TF expression is associated with a worse oncologic outcome [12-14].

Fibrinogen, the most abundant plasma coagulation factor, is a key protein in coagulation pathway, clot formation, and wound healing, and is required for platelet aggregation, which is the final step in the coagulation cascade. It has previously been suggested that fibrinogen and platelets have a role in carcinogenesis, and their levels are associated with tumor progression, metastasis, and angiogenesis [15-17]. Furthermore, recent studies have shown that elevated fibrinogen plasma levels and platelet counts are a useful

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prognostic parameter for recurrence and overall survival [18,19].

Although previous studies suggested a close relationship between colorectal cancer and hypercoagulability, TF was not fully investigated in colorectal cancer. In this study, therefore, we examined the TF expression in patients with non-metastatic colorectal cancer who underwent curative resection. We then analyzed the relationship between TF expression and other coagulation abnormalities, including thrombocytosis and hyperfibrinogenemia, as well as the prognostic factors of tumor recurrence to determine the prognostic role of TF expression.

METHODS

Data pertaining to a total of 305 patients with stage II or III colorectal cancer who underwent curative resection between 2000 and 2007 at The University of Tokyo Hospital were collected retrospectively from a prospectively maintained database. Patients who had a synchronous extracolonic tumor, apparent acute inflammatory disease, severe liver disease, distant metastasis, or preoperative chemotherapy or radiotherapy were excluded. The study was approved by the Institutional Review Board of The University of Tokyo Hospital and performed in accordance with the principles of the Declaration of Helsinki. The informed consent was waived.

After curative surgery, patients were followed up postoperatively every 3 months for the first 2 years, every 6 months for the next 3 years, and once annually thereafter. Patients were clinically examined and tumor markers were measured at each visit. Full colonoscopy was performed 1 year after surgery, and then once every 3 years. Computed tomography scans were obtained on the basis of individual follow-up regimens (generally every 6–12 months). Recurrences were confirmed radiologically or by biopsy. Locoregional recurrence was defined as tumor regrowth within the pelvis, perineum, or anastomosis site; whereas, distant metastasis was defined as any other recurrence outside of the pelvis.

TF immunohistochemistry

Immunohistochemical staining was performed using formalin-fixed, paraffin-embedded, 3 μ m-thick colorectal cancer tissue sections. These tissues were deparaffinized in xylene and rehydrated in graded alcohols. For antigen retrieval, sections were boiled in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes in a microwave oven. Endogenous peroxidase activity was quenched using methanol and 3% hydrogen peroxide solution for 15 minutes. After washing with phosphate-buffered saline (PBS), the sections were incubated overnight at 4°C with a rabbit polyclonal anti-TF antibody (FL-295, dilution 1:75; Santa Cruz Biotechnology, Dallas, TX, USA). After washing in PBS, the sections were incubated with a secondary anti-rabbit biotinylated antibody (Santa Cruz Biotechnology) for 20 minutes at room temperature. After washing again in PBS, these slides were treated with peroxidase-conjugated streptavidin for 10 minutes and developed by immersion in a 0.01% hydrogen peroxide solution and diaminobenzidine tetrahydrochloride. Light counterstaining was performed with Mayer's hematoxylin.

Immunoreactivity was assessed by Y.K and K.K who had no knowledge of the associated clinical background. Immunoreactivity of TF was classified as “high” when the cytoplasmic or membrane staining was at least of moderate intensity and occupied more than 75% of the cancer cells in each section.

Statistical analysis

Statistical calculations were performed using statistical software, SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Continuous data were analyzed by comparing the mean value of each variable between groups by using the Student t-test. Categorical variables were compared using Fisher exact test or the chi-square test, as appropriate. The probability of disease-free survival was estimated using the Kaplan-Meier method. The log-rank test was employed to compare survival between groups. A P-value of <0.05 was considered statistically significant.

Fig. 1. Immunohistochemical staining with an anti-tissue factor antibody. (A) Strong immunoreactivity (B) moderate immunoreactivity, and (C) no immunoreactivity. Magnification, x200.

RESULTS

A total of 297 patients were eligible for inclusion in this study. TF expression was high in 257 of the 297 patients (86.5%) (Fig. 1). Table 1 summarizes the associations between TF expression and the clinicopathologic characteristics of the patients. TF expression did not differ significantly according to age, sex, histologic differentiation, depth of invasion, or lymph node metastasis. Further, high TF expression was not significantly associated with the platelet count ($P = 0.180$) or fibrinogen level ($P = 0.281$).

The associations between other coagulation abnormalities, thrombocytosis (platelet count $\geq 370 \times 10^3/\mu\text{L}$) and hyperfibrinogenemia (fibrinogen level $\geq 350 \text{ mg/dL}$), and the clinicopathologic characteristics of patients are summarized in Table 2. In both comparisons, advanced tumor depth was significantly associated with thrombocytosis ($P = 0.024$) and hyperfibrinogenemia ($P = 0.001$).

The median clinical follow-up was 69 months (interquartile range, 26–112 months). A total of 73 patients experienced tumor recurrences during the follow-up period, but this occurred more frequently in the high TF expression group than in the low TF expression group (67 patients [26.1%] vs. 6 patients [15.0%], $P = 0.167$). Fig. 2 shows the Kaplan-Meier survival curves of patients according to TF expression. The 5-year disease-free survival rate in patients with high TF expression was 72.3%, compared to 83.9% for those with low TF expression although this difference was not

Table 1. Correlations between tissue factor expression and clinicopathologic characteristics

Characteristic	Low TF expression (n = 40)	High TF expression (n = 257)	P-value
Age (yr)	67.6 $\pm$ 9.9	67.7 $\pm$ 11.5	0.923
Sex			0.084
Male	19 (47.5)	160 (62.3)	
Female	21 (52.5)	97 (37.7)	
Tumor location			0.343
Colon	26 (65.0)	188 (73.2)	
Rectum	14 (35.0)	69 (26.8)	
Histologic differentiation			0.280
Well or moderately	4 (10.0)	14 (5.4)	
Others	36 (90.0)	243 (94.6)	
Lymphatic invasion			0.853
Negative	29 (72.5)	178 (69.3)	
Positive	11 (27.5)	79 (30.7)	
Venous invasion			0.718
Negative	14 (35.0)	82 (31.9)	
Positive	26 (65.0)	175 (68.1)	
Depth of tumor invasion			0.153
T1&T2	5 (12.5)	14 (5.4)	
T3&T4	35 (87.5)	243 (94.6)	
Lymph node metastasis			0.172
Negative	17 (42.5)	142 (55.3)	
Positive	23 (57.5)	115 (44.7)	
Platelet count ($\times 10^3/\mu\text{L}$)	289.7 $\pm$ 106.0	269.2 $\pm$ 86.0	0.180
Fibrinogen (mg/dL)	363.3 $\pm$ 76.0	379.2 $\pm$ 85.3	0.281
Disease recurrence	6 (15.0)	67 (26.1)	0.167

Values are presented as mean $\pm$ standard deviation or number (%).
TF, tissue factor.

Table 2. Correlation between platelet count or fibrinogen level and clinicopathologic characteristics

Characteristic	Platelet count ($\times 10^3/\mu\text{L}$)		P-value	Fibrinogen (mg/dL)		P-value
	≥ 370 (n = 36)	< 370 (n = 260)		≥ 350 (n = 168)	< 350 (n = 111)	
Age (yr)	64.8 $\pm$ 10.6	68.1 $\pm$ 11.3	0.108	-	-	-
Sex			0.047			0.619
Male	16 (44.4)	162 (62.3)		97 (57.7)	68 (61.3)	
Female	20 (55.6)	98 (37.7)		71 (42.3)	43 (38.7)	
Tumor location			0.697			0.784
Colon	25 (69.4)	188 (72.3)		121 (72.0)	82 (73.9)	
Rectum	11 (30.6)	72 (27.7)		47 (28.0)	29 (26.1)	
Histologic differentiation			0.252			0.788
Well or moderately	32 (88.9)	246 (94.6)		10 (6.0)	5 (4.5)	
Others	4 (11.1)	14 (5.4)		158 (94.0)	106 (95.5)	
Lymphatic invasion			0.847			0.596
Negative	26 (72.2)	180 (69.2)		119 (70.8)	75 (67.6)	
Positive	10 (27.8)	80 (30.8)		49 (29.2)	36 (32.4)	
Venous invasion			0.852			0.603
Negative	11 (30.6)	85 (32.7)		57 (33.9)	34 (30.6)	
Positive	25 (69.4)	175 (67.3)		111 (66.1)	77 (69.4)	
Depth of tumor invasion			0.024			0.001
T1&T2	0	19 (7.3)		4 (2.4)	14 (12.6)	
T3&T4	36 (100)	241 (92.7)		164 (97.6)	97 (87.4)	
Lymph node metastasis			0.109			0.327
Negative	24 (66.7)	134 (51.5)		94 (56.0)	55 (49.5)	
Positive	12 (33.3)	126 (48.5)		74 (44.0)	56 (50.5)	

Values are presented as mean $\pm$ standard deviation or number (%).

Fig. 2. Kaplan-Meier curves for disease-free survival according to the extent of tissue factor expression. (A) Tissue factor (TF) $\geq 75\%$ vs. 50%–75% vs. $< 50\%$ and (B) TF $\geq 75\%$ vs. $< 75\%$ ($P=0.074$).

Table 3. Univariate analysis for disease-free survival

Characteristic	No.	5-Year DFS (%)	P-value
Age (yr)			0.687
< 60	64	73.5	
≥ 60	233	74.0	
Sex			0.826
Male	179	74.0	
Female	118	73.6	
Histologic differentiation			0.092
Well or moderately	278	75.1	
Others	19	53.8	
Lymphatic invasion			0.012
Negative	207	82.7	
Positive	90	65.3	
Venous invasion			< 0.001
Negative	96	87.7	
Positive	201	67.1	
Depth of tumor invasion			0.475
T1&T2	19	65.2	
T3&T4	278	74.5	
Lymph node metastasis			< 0.001
Negative	159	83.8	
Positive	138	63.4	
Platelet count ($\times 10^3/\mu\text{L}$)			0.479
≥ 370	36	79.3	
< 370	260	73.0	
Fibrinogen (mg/dL)			0.234
≥ 350	168	73.3	
< 350	111	77.7	
Tissue factor expression			0.074
Negative	40	83.9	
Positive	257	72.3	

DFS, disease-free survival.

Table 4. Cox proportional hazards model for disease-free survival

Variable	HR (95% CI)	P-value
Histologic differentiation (differentiation vs. others)	2.911 (1.308–6.481)	0.009
Lymphatic invasion (positive vs. negative)	1.227 (0.759–1.983)	0.405
Venous invasion (positive vs. negative)	2.784 (1.431–5.417)	0.003
Lymph node metastasis (positive vs. negative)	2.497 (1.499–4.158)	< 0.001
Tissue factor expression (positive vs. negative)	2.446 (1.054–5.674)	0.037

HR, hazard ratio; CI, confidence interval.

statistically significant ($P=0.074$). When we sub-classified the proportion of TF positive cells in the section as $< 50\%$, 50%–75%, and $\geq 75\%$, TF expression $< 50\%$ was associated with a better 5-year disease-free survival rate than TF expression 50%–75% (83.6% vs. 78.2%, $P=0.428$) or TF expression $\geq 75\%$ (83.6% vs. 70.8%, $P=0.060$), although this difference was also not statistically significant (Fig. 2).

In a univariate analysis, lymphatic invasion ($P=0.012$), venous invasion ($P<0.001$), and lymph node metastasis ($P<0.001$) were significantly associated with disease recurrence. Histologic differentiation and TF expression showed the trend, but these were not statistically significant ($P=0.092$ and $P=0.074$, respectively). However, thrombocytosis and hyperfibrinogenemia were not significantly associated with recurrence ($P=0.479$ and $P=0.234$, respectively) (Table 3). Variables in the univariate analysis with a P-value < 0.2 were included in the multivariate analysis. In the multivariate Cox hazard model, undifferentiated histologic type (hazard ratio [HR], 2.911; 95% confidence interval [CI], 1.308–6.481; $P=0.009$), venous invasion (HR, 2.784; 95% CI, 1.431–

5.417; $P = 0.003$), lymph node metastasis (HR, 2.497; 95% CI, 1.499–4.158; $P < 0.001$), and high TF expression (HR, 2.446; 95% CI, 1.054–5.674; $P = 0.037$) were all found to be significantly associated with disease-free survival (Table 4).

DISCUSSION

To assess the risk of relapse among non-metastatic colorectal cancer patients who have undergone curative resection would be of great clinical implications. In this study, we evaluated the prognostic role of coagulation factors, including TF expression, the fibrinogen level, and the platelet count in patients with stage II or III colorectal cancer. We found a significant association between high TF expressions in tumor cells and a high tumor recurrence rate in multivariate analysis. However, TF expression was not correlated with other coagulation abnormalities, including an elevated fibrinogen level and platelet counts, and these were not significantly associated with tumor recurrence.

TF is a 47-kDa transmembrane glycoprotein that is essential for hemostasis. It binds to the coagulation serine protease factor VII/VIIa (FVII/VIIa) to form a protein complex that functions as the primary initiator of coagulation. The TF/FVIIa complex activates both factor X and factor IX and leads to thrombin generation and fibrin formation, both of which are required for physiologic hemostasis. Thrombin also activates platelets through the cleavage of protease-activated receptors [5,6]. In addition to its role in hemostasis, TF has a possible role in cancer growth, metastasis, and recurrence. TF is constitutively expressed in various extravascular cells, but it is also aberrantly expressed in cancer cells and endothelial cells within tumors. It induces angiogenesis by up-regulating vascular endothelial growth factor and down-regulating the angiogenesis inhibitor thrombospondin, in a mechanism that is independent of coagulation [20]. Thus, TF may also contribute to cancer coagulopathy, intratumoral angiogenesis, and tumor progression [8–11]. This is further supported by the findings that a selective reduction in TF expression in colorectal cancer cells using small interfering RNA dramatically reduced tumor growth in a mouse model of malignancy [21].

Previous studies have reported significant association between the immunohistochemical localization of the TF antigen in tumors and poor oncologic outcomes in colorectal cancer. Seto et al. [9] showed that TF expression in colorectal cancer was an independent risk factor for hepatic metastasis and poor prognosis in 67 colorectal cancer patients. Shigemori et al. [22] reported that TF expression was detected in 57% of 79 colorectal tumors and in 88% of 17 liver metastatic tumors from primary colorectal cancer. Findings from these two studies suggest that the immunoreactivity of

TF is closely related to metastasis in colorectal cancer. In agreement with these results, our study also showed that high TF expression in colorectal cancer cells was an independent prognostic factor for disease recurrence after adjustment for confounding variables, such as histologic differentiation, venous invasion, and lymph node metastasis in stage II or III colorectal cancer.

There is no standard classification of high TF expression in immunohistochemical staining. Some authors have suggested that high or positive TF expression should be determined on the basis of intensity of staining [23], whereas other authors have suggested the area of the section staining positive for TF should be the basis for determining a high or positive TF expression [15,24]. In this study, we mainly applied the latter, and considered a tumor to be high TF expression when more than 75% of the cancer cells expressed TF. We also supplement our classification for high TF expression by adding to the intensity of staining (at least moderate intensity). In this study, the proportion of patients with high TF expression (86.5%) was higher than reported in previous studies (approximately 50%) [14,23,24]. The discrepancy between our findings for high TF expression and those of previous studies might be explained by our inclusion of only stage II or III colorectal cancer patients, most of whom had T3 or T4 tumors (278 of 297 patients), because previous studies reported that high TF expression was associated with advanced clinical stage, tumor depth, and tumor size [25]. This assumption also might explain the lack of any apparent relationship between TF expression and patient's clinicopathologic characteristics in this study. Mostly advanced tumor stage (T3 or T4) in this study may have made it more difficult to draw the statistically significant associations between TF expression and those factors.

None of the previous studies addressing TF expression in colorectal cancers investigated the relationship between TF expression and thrombocytosis or hyperfibrinogenemia. However, contrary to our initial hypothesis, we failed to find associations among the coagulation factors. Also, we did not observe any significant association between thrombocytosis or hyperfibrinogenemia and disease recurrence. We consider it also related to selection bias, because thrombocytosis and hyperfibrinogenemia are also known to be related to clinical stage, tumor depth and size [16,18,19]. It was therefore difficult to find a statistically significant relationship between TF expression, serum fibrinogen levels and platelet counts. However, thrombocytosis or hyperfibrinogenemia is known to be a poor prognostic factor in colorectal cancer. Therefore, further large cohort study is required to evaluate this relationship.

We found that colorectal patients with high TF expression showed a poorer oncologic outcome than those with low TF expression, suggesting that TF can be a potential target in cancer therapy. Various TF-directed therapeutic agents are being devel-

oped, including TF antagonists such as recombinant TF pathway inhibitors and anti-TF antibodies, and other agents that act indirectly on TF expression, such as vitamin D3, retinoids, and heparin [26-28]. Preclinical studies have shown that some of these agents have antitumor and antiangiogenic effects [29]. TF-targeting therapeutic agents have been tested for the treatment of both cancer and non-cancerous diseases. The results of these trials indicate that these agents can destroy tumor vessels by targeting tumor vascular endothelial cells. When administered systemically, these agents can penetrate tumor tissue and kill the tumor cells through leaks in the tumor vasculature. In a mouse model of pulmonary metastasis, inhibition of TF with a variety of agents, such as anti-TF antibodies and a TF pathway inhibitor, reduced tumor metastasis [30].

This study had several limitations, including the selection biases that are inherent in any retrospective analysis. However, we included a relatively high number of patients with stage II or III colorectal cancer, and the follow-up period was relatively long. Because we only included patients with stage II or III colorectal cancer, we did not observe any apparent relationship between TF expression and patient characteristics. However, a number of stage II or III colorectal cancer patients experienced tumor recurrence and death. There is a clinical implication in evaluating prognostic factors for disease recurrence in these patients and it would help to select patients who would benefit from aggressive adjuvant treatment or molecular targeted therapy. Nevertheless, further study is required to evaluate the implication of TF expression in all stages of colorectal cancer. The second limitation was the accuracy with which TF expression was evaluated. However, two pathologists assessed immunoreactivity without knowing the clinical background of the patients, and a Cohen's kappa test for concordance between the two pathologists gave a value of 0.746, indicating good agreement between them. Therefore, our results suggest that immunohistochemistry is a reliable tool for assessing TF expression in tumors, which in turn could allow the prediction of the oncologic outcome in cancer patients.

In this study, we showed that high levels of TF expression in colorectal cancer cells was associated with disease recurrence in patients with stage II or III colorectal cancer. TF expression could be considered as a prognostic indicator for patients with non-metastatic colorectal cancer. Therefore, the determination of the TF expression status may provide additional prognostic information. However, further studies are needed to establish the underlying mechanism relating TF expression with oncologic outcome. Further studies are also needed to determine the true clinical usefulness of these findings and identify possible roles for TF as a clinical marker and therapeutic target.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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