Tissue Factor Expression Correlates with Tumor Angiogenesis and Invasiveness in Human Hepatocellular Carcinoma

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ABSTRACT

Purpose: Recent studies have shown that tissue factor (TF) may be involved in tumor angiogenesis and metastasis. The role of TF in hepatocellular carcinoma (HCC) was unknown. This study evaluated whether TF expression correlates with microvesSEL density (MVD), vascular endothelial growth factor (VEGF) expression, tumor invasiveness, and prognosis in human HCC.

Experimental Design: Tissue samples were obtained from 58 specimens of resected HCC. Immunohistochemical expression of TF was examined, and tumor MVD was evaluated using CD34 as the endothelial marker. TF and VEGF protein levels in the tumor cytosol were quantified by ELISA. Clinicopathologic and follow-up data of patients were prospectively collected.

Results: The immunohistochemical expression of TF in the tumors correlated significantly with tumor MVD (P = 0.002). The median cytosolic TF protein level in the tumors was 720 pg/mg total protein (range, 67–2406 pg/mg total protein). A significant positive correlation was found between TF and VEGF levels in the tumor cytosol (r = 0.475, P < 0.001). High tumor cytosolic TF level was associated with venous invasion (P = 0.004), microsatellite nodules (P = 0.024), unencapsulated tumor (P = 0.007), and advanced tumor stage (P = 0.010). A higher than median tumor cytosolic TF level was an independent predictor of poor survival (risk ratio, 1.836; 95% confidence interval 1.130–5.312, P = 0.023).

Conclusions: This study shows that TF is related to tumor angiogenesis and invasiveness in HCC. Evaluation of tumor TF expression may be useful as a prognostic indicator in patients with HCC.

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INTRODUCTION

TF,1 a Mr 47,000 glycoprotein, is the principal physiological initiator of blood coagulation (1). It triggers the extrinsic pathway of blood coagulation by binding factor VII. TF is expressed by human cancers and is a major procoagulant that causes a hypercoagulable state in cancer patients (2). Experimental studies have demonstrated that TF also plays an important role in tumor invasion and metastasis (3, 4). Expression of TF was found to have a significant correlation with metastatic potential in human lung (5), breast (6), and colorectal (7, 8) carcinomas. Although TF may enhance cancer cell metastasis by influencing cellular migration and adhesion (9), an important mechanism through which TF promotes tumor growth and metastasis is by inducing tumor angiogenesis (10). The neovessels in a tumor not only provide oxygen and nutrients for tumor growth, but they also provide the route for tumor cell invasion into the circulation. Recent studies have demonstrated that tumor expression of TF is significantly related to tumor angiogenesis in lung (11), prostate (12), and colorectal (13) carcinomas. The angiogenic property of TF is mediated at least, in part, through the up-regulation of VEGF (13–15).

HCC is characterized by active neovascularization. There is accumulating evidence that tumor angiogenesis plays an important role in the progression and metastasis of HCC (16–18). The role of TF in the angiogenesis and tumor progression of HCC remains unknown because no studies on TF expression in HCC have ever been published. Hence, we conducted a prospective study to evaluate the expression of TF and its correlation with angiogenesis and tumor invasiveness in HCC.

MATERIALS AND METHODS

Patients and Tissue Specimens. Between January 1998 and September 2000, 58 patients who underwent curative hepatic resection for HCC at the Department of Surgery of the University of Hong Kong at Queen Mary Hospital (Hong Kong, China) were recruited into the study. Curative resection was defined as complete clearance of the tumor both macroscopically and microscopically. The study was approved by the Ethics Committee of our institution and informed consent was obtained from the patients. Tumor specimens were obtained immediately after surgical resection. A part of the tumor tissues was immediately fixed in formalin, then paraffin-embedded and used for immunohistochemical studies of TF expression and MVD. Another part of the tumor tissues was snap frozen with liquid nitrogen and stored at −70°C for study of cytosolic concentration of TF and VEGF in the tumor.

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**Immunohistochemical Staining of TF.** Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase complex method. Formalin-fixed, paraffin-embedded sections of 4 μm were deparaffinized in xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase was blocked by treating the sections with 3% hydrogen peroxide in methanol for 10 min. The sections were then subjected to antigen retrieval by microwave treatment for 10 min. Afterward, the sections were incubated with normal goat serum for 30 min at 37°C and then with 1:100 diluted TF mouse antihuman monoclonal antibody (American Diagnostica, Inc., Greenwich, CT) for 1 h at 37°C. In the negative control experiment, the tissue sections were incubated with normal mouse immunoglobulin instead of the primary antibody. Sections were then incubated with 1:100 diluted biotin-conjugated goat antimouse immunoglobulin secondary antibody (Zymed Laboratory Inc., San Francisco, CA) for 1 h at 37°C and developed in 3,3′-diaminobenzidine tetrchloride (Dako, Carpinteria, CA). The immunoreactivity of TF was classified as high if >50% of the tumor cells were stained positively and low if <50% of the tumor cells were stained positively.

**Evaluation of MVD.** Details of MVD determination have been described in a previous study by the authors (19). Briefly, tissue sections were immunostained with human CD34 monoclonal antibody (BioGenex, San Ramon, CA). At low power field (×40), the tissue sections were screened, and five areas with the most intense neovascularization (hot spots) were selected. Microvessel counts of these areas were performed at HPF (×200). To reduce observer-related variation, counting of the microvessels was performed with a computer image analyzer (MetaMorph Imaging System version 3.0; Universal Imaging Corporation, West Chester, PA), which is an integrated system of a Windows-based software especially designed for immunohistochemical analysis. Any positively stained endothelial cell or endothelial-cell cluster that was clearly separated from adjacent microvessels, tumor cells, and connective elements was counted as one microvessel, irrespective of the presence of a vessel lumen. An automated microvessel count/field was performed in each hot spot, and the mean microvessel count of the five most vascular areas was used as the MVD, which was expressed as the absolute number of microvessels/HPF.

**Quantitation of Cytosolic TF and VEGF.** Cytosol was obtained by homogenization of tumor tissues as described in previous studies by other authors (20, 21). Briefly, fresh tumor tissues were homogenized in 4 volumes of homogenization buffer [containing 10 mM Tris-hydrochloride (pH 7.4), 1.5 mM EDTA, 5 mM disodium molybdate, 100 ml/liter glycerol, and 1 mM monothioglycerol] and one-third volume of 1.0-mm glass beads (Biospec Products, Batleville, OK). Homogenization was performed at 4200 rpm for 10 s using a Mini-beadbeater (Biospec Products). Homogenates were then centrifuged at 20,000 × g at 4°C for 10 min. The supernatants were used for quantitative assay of TF, VEGF, and total protein.

TF and VEGF content in the tumor cytosol was measured by ELISA using Immunobind TF ELISA kit (American Diagnostica, Inc.) and Quantikine human VEGF ELISA kit (R&D Systems, Minneapolis, MN), respectively. Total protein content was measured using Bio-Rad total protein assay (Bradford, Hercules, CA). The concentrations of TF and VEGF in the cytosol were expressed as pg protein/mg total protein.

**Clinicopathologic Data.** Clinicopathologic data of all patients (Table 1) were prospectively collected in a computerized database. Tumor was graded according to the criteria described by Edmonson and Steiner (22) and staged according to the pathological TNM classification (23). The investigators who performed the laboratory studies of TF and VEGF expression were blinded of the clinicopathologic data. All patients were followed in the outpatient clinic with regular surveillance for recurrence by serum AFP level and contrast-enhanced computerized tomography scan.

**Statistical Analysis.** Continuous data were expressed as median and range and compared between groups using the Mann-Whitney U test. Categorical variables were compared using the χ² test (or Fisher’s exact test where appropriate). Paired comparison of continuous data were performed using the Wilcoxon signed ranks test. Correlation between continuous variables was tested using the Spearman correlation coefficients (r). Patient survival was calculated using the Kaplan-Meier method and compared using the log-rank test. Multivariate analysis of prognostic factors for survival was performed using a Cox stepwise regression model. All statistical analyses were performed using a statistical software (SPSS 9.0 for Windows; SPSS, Inc., Chicago, IL). Ps < 0.05 were considered statistically significant.

**RESULTS**

**Correlation between TF Immunoreactivity and MVD.** Immunohistochemical staining of the tumor sections showed that TF was expressed by HCC tumor cells in all of the tumors studied to a variable extent (Fig. 1), with high immunoreactivity in 22 patients and low immunoreactivity in 36 patients. Specific staining of capillary-like vessels by anti-CD34 was observed in all tumor specimens (Fig. 2). The median tumor MVD was 44.4/HPF (range, 4.8–131.2). The MVD was significantly higher in tumors with high immunoreactivity for TF than in tumors with low immunoreactivity for TF (median, 65.0 versus 33.6/HPF, P = 0.002; Fig. 3).

**Correlation between Tumor Cytosolic TF and VEGF Levels.** The median tumor cytosolic TF level by ELISA was 720 pg/mg total protein (range, 67–2406). Higher TF levels...
were observed in tumors with high immunoreactivity for TF compared with those with low immunoreactivity for TF (median, 938 versus 528 pg/mg total protein, $P = 0.012$). Cytosolic TF levels were also determined in the adjacent nontumorous liver. The tumor TF levels were significantly higher than the nontumorous TF levels (median, 306 pg/mg total protein; range 23–910, $P = 0.026$).

The cytosolic VEGF levels in the tumors (median, 168.4 pg/mg total protein; range 53–1568) were significantly higher than the nontumorous VEGF levels (median, 18.5 pg/mg total protein; range 6–70, $P = 0.0002$).

**Fig. 1** TF immunostaining (brownish staining) in a tumor section (original magnification, ×200).

**Fig. 2** Intensive staining of microvessels by anti-CD34 immunostaining (brownish staining) in a tumor section (original magnification, ×200).
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tumor cytosolic TF levels between tumors categorized by vari-

Table 2 shows comparisons of the Tumor Characteristics.

<table>
<thead>
<tr>
<th>Tumor size</th>
<th>Tumor cytosolic TF level* (pg/mg total protein)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤5 cm</td>
<td>676 (155–2383)</td>
<td>0.426</td>
</tr>
<tr>
<td>&gt;5 cm</td>
<td>852 (67–2406)</td>
<td></td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>552 (155–960)</td>
<td>0.002</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>960 (282–2406)</td>
<td>0.143</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>1002 (185–2300)</td>
<td></td>
</tr>
<tr>
<td>Venous invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>552 (67–1434)</td>
<td>0.004</td>
</tr>
<tr>
<td>Present</td>
<td>1002 (185–2406)</td>
<td></td>
</tr>
<tr>
<td>Microsatellite nodules</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>552 (67–2383)</td>
<td>0.024</td>
</tr>
<tr>
<td>Present</td>
<td>960 (211–2406)</td>
<td></td>
</tr>
<tr>
<td>Tumor encapsulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>960 (185–2406)</td>
<td>0.007</td>
</tr>
<tr>
<td>Present</td>
<td>529 (67–1736)</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I &amp; II</td>
<td>552 (67–2383)</td>
<td>0.010</td>
</tr>
<tr>
<td>III &amp; IVA</td>
<td>960 (185–2406)</td>
<td></td>
</tr>
</tbody>
</table>

* Tumor cytosolic TF levels expressed as median with range in parentheses.

pg/mg total protein; range, 1.0–3626) were also significantly higher than those in the nontumorous livers (median, 29.5 pg/mg total protein; range, 3.6–75.0, P = 0.004). Positive correlation was observed between TF and VEGF levels (r = 0.475, P < 0.001) and between TF levels and MVD (r = 0.522, P < 0.001) in the tumors.

Relationship between Tumor Cytosolic TF Levels and Tumor Characteristics. Table 2 shows comparisons of the tumor cytosolic TF levels between tumors categorized by various characteristics. Cytosolic TF levels were significantly higher in moderately differentiated or poorly differentiated tumors than in well-differentiated tumors, but no significant difference was found between the former two groups. Higher tumor TF levels were associated with the presence of venous invasion (P = 0.004), presence of microsatellite nodules (P = 0.024), absence of tumor capsule (P = 0.007), and advanced TNM stage (P = 0.010). Tumor TF levels were not significantly different between tumors ≤5 cm and those >5 cm. There was no significant correlation between tumor TF levels and either tumor size (P = 0.834) or serum AFP level (P = 0.333).

Prognostic Significance of Tumor TF Levels. The 58 patients were categorized into low (n = 29) and high (n = 29) tumor TF levels using the median tumor cytosolic TF level of 720 pg/mg total protein as the cutoff point. By the time of analysis, the patients had been followed for a median period of 40 months (range, 20–54 months) after surgery. Patients with low tumor TF levels had significantly better survival results than those with high tumor TF levels (P = 0.015, Fig. 4). The cumulative 1-, 2-, and 3-year survival rates of patients with low tumor TF levels were 90, 81, and 76%, respectively, whereas the corresponding survival rates for patients with high TF levels were 68, 60, and 54%, respectively. By the time of data analysis, 6 patients in the low tumor TF level group and 13 patients in the high tumor TF level group had died. All of the deaths were related to tumor recurrence. Another 3 patients in the low tumor TF level group and 5 patients in the high tumor TF group had developed recurrence but were alive.

When tumor TF level was entered into a multivariate analysis taking into account other clinicopathologic factors known to influence the prognosis after resection of HCC (serum bilirubin, serum AFP, nontumorous liver status, tumor size, venous invasion, microsatellite nodules, tumor encapsulation, and tumor grading), tumor TF level (risk ratio, 1.836; 95% confidence interval, 1.130–5.312, P = 0.023) and venous invasion (risk ratio, 2.318; 95% confidence interval, 1.250–4.386, P = 0.008) were the only significant prognostic factors.
DISCUSSION

HCC is a highly malignant tumor with a propensity for vascular invasion and metastasis. Hepatic resection is the treatment of choice for HCC, but the prognosis after resection remains unsatisfactory because of a high incidence of recurrence related to tumor metastasis (24). Tumor angiogenesis is an important determinant of invasiveness and progression of HCC (16–19). However, little is known of the regulatory mediators of angiogenesis in HCC. There is some evidence that VEGF is an important angiogenic mediator of HCC (17, 25–27). The role of other angiogenic factors in HCC is less clear.

TF has been recently recognized to be capable of inducing angiogenesis, which is mediated at least in part through up-regulation of VEGF (10, 14, 15). The elucidation of the angiogenic activity of TF is interesting because it underlines an intriguing relationship between hemostasis and angiogenesis (28). Such a relationship is of physiological importance in that proteins generated by the coagulation system in response to tissue damage can enhance angiogenesis for tissue repair. TF also appears to make an important contribution to tumor angiogenesis, which represents an imbalance in the normal mechanisms that allow organized healing after injury (29). The role of TF in tumor angiogenesis has been recently studied in a few human cancers (11–13). To our knowledge, this is the first study that evaluates the expression of TF and its relationship with angiogenesis in HCC.

By immunohistochemical staining, we showed that TF was expressed by HCC tumor cells in all of the 58 specimens. However, there was a wide variation in TF expression among different tumors, which was confirmed by the quantitative measurement of TF in the tumor cytosol using ELISA. We observed a significant correlation between TF expression and tumor MVD, suggesting that TF is an important regulator of angiogenesis in HCC. This finding agrees with the results reported in other cancers (11–13). To elucidate the possible pathway through which TF is involved in the angiogenesis of HCC, we evaluated its relationship with VEGF. VEGF is an endothelial-cell-specific mitogen and is a major inducer of angiogenesis in human cancers (30). Our study showed a positive correlation between TF and VEGF levels in the tumor cytosol. TF may regulate tumor angiogenesis in HCC via up-regulation of VEGF as in other cancers (13–15).

Correlation between tumor TF levels and histopathological features revealed a significant association between high TF expression and the presence of venous invasion, presence of microsatellite nodules, absence of tumor capsule, and advanced TNM stage. All these features indicated invasive or advanced cancer, and they are associated with a poorer prognosis after resection of HCC because of an increased risk of intrahepatic or extrahepatic metastasis (24, 31–34). Features of tumor invasiveness such as venous invasion and microsatellite nodules are known to be associated with large tumor size (34). In our analysis, there was no significant correlation between tumor TF levels and tumor size. Tumor TF expression may influence tumor invasiveness independent of tumor size. Instead, TF expression was up-regulated in moderately or poorly differentiated HCC compared with well differentiated HCC. Previous studies in pancreatic carcinoma and glioma also showed a higher TF expression in higher grade tumors (35, 36).

In this study, patients with a high tumor TF level had a significantly worse prognosis than those with a low tumor TF level. This finding additionally supports a significant role of TF in the aggressiveness of HCC. A recent study has shown that tumor TF expression was a prognostic factor independent of conventional pathological features in breast cancer (6). Another study found that TF expression was an independent predictor of liver metastasis in colorectal carcinoma (8). In our multivariate analysis, TF expression was also an independent prognostic factor. Evaluation of tumor TF expression in the surgical specimens may have a potential value for prognostication in HCC. Such information may be useful in the consideration of adjuvant therapies for patients with invasive tumors. A recent study in breast cancer patients has demonstrated that plasma TF concentration correlated with tumor expression of TF, suggesting that plasma TF concentration may have a prognostic value in cancer patients (6). This is in agreement with the prognostic value of circulating levels of other angiogenic factors such as VEGF demonstrated in cancer patients (37). Previous studies by the authors have shown that serum VEGF levels correlated with tumor expression of VEGF in HCC patients (38), and preoperative serum VEGF levels were predictive of venous invasion in HCC (17). We did not measure the serum TF levels in the cohort of patients in this study. It is worthwhile to further investigate the prognostic significance of circulating TF levels in HCC patients, which may be particularly useful in the management of patients with unresectable HCC.

Our finding of an apparent association of TF with angiogenesis and tumor invasiveness may have a therapeutic implication in addition to its prognostic value. Treatment targeting tumor angiogenesis is now in clinical trials (30, 39). Antiangiogenic therapy holds considerable promise in the treatment of HCC because of the vascularity of this tumor (40). Antiangiogenic therapy may be useful as an adjuvant therapy in patients undergoing resection of HCC. Therapy targeting VEGF has been shown to inhibit the growth of HCC (41). TF may be a novel target for antiangiogenic therapy in HCC. Blocking of TF activity by monoclonal antibodies could inhibit tumor metastasis in experimental models (42). TF pathway inhibitor, a natural inhibitor of TF mediated coagulation, has been shown to inhibit metastasis in an animal model of melanoma (43). Tumor TF expression can also be suppressed by pentoxifylline (44) and retinoic acid (45). On the basis of our data, it is worthwhile to explore the efficacy of therapies targeting TF for the treatment of HCC.

In conclusion, this study shows that the expression of TF in HCC is related to tumor angiogenesis and invasiveness. In addition to its potential prognostic value, this novel finding may provide insight into a new therapeutic strategy for HCC by inhibiting TF expression.

REFERENCES


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