Serum Tenascin-C as a Potential Predictive Marker of Angiogenesis in Non-small Cell Lung Cancer

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Abstract. Background: Tenascin (Tn)-C is an extracellular matrix protein that is involved in tissue interactions during fetal development and onogenesis. However, the role of serum Tn-C in non-small cell lung cancer (NSCLC) has not been clarified. Patients and Methods: In this study, we determined the serum levels of Tn-C among NSCLC patients who underwent surgery, as well as other factors implicated for angiogenesis, to address the clinical implications in NSCLC. Results and Conclusion: The median concentration of serum Tn-C in NSCLC patients was slightly higher than that of normal controls, but this difference was not statistically significant. There was a positive correlation between serum Tn-C levels and microvessel density (MVD), serum osteopontin (OPN) and vascular endothelial growth factor (VEGF). In contrast, there was no correlation between serum Tn-C levels and serum carcinoembryonic antigen (CEA) and sialyl lewis-X (SLX) levels. The overall survival of patients with low Tn-C levels (<96 ng/ml) was significantly greater than that of patients with high Tn-C levels (≥96 ng/ml). Intratumoral Tn-C expression was co-localized with expression of microvessels in the stroma of the cancer cells by immunohistochemical analysis. Moreover, enhanced in vitro migration of human umbilical vascular endothelial cells (HUVEC) was induced by recombinant Tn-C. Collectively, Tn-C may play an important role in angiogenesis of patients with NSCLC, and the determination of serum Tn-C may be useful in predicting intratumoral vasculature and patients’ prognosis.

Primary lung cancer is the leading cause of cancer-related deaths in Japan (1, 2). Non-small cell lung cancer (NSCLC) is the most frequent type of lung cancer, and is treated by surgical resection for patients diagnosed as early stage carcinoma. Unfortunately, more than 50% of NSCLC patients who undergo surgery will eventually experience local relapse or distant metastasis. This high metastatic potential of NSCLC may be due to the active angiogenesis of the tumors, in which many angiogenic factors such as vascular endothelial growth factor (VEGF) and osteopontin (OPN) play an important role (3, 4). Adjuvant combination chemotherapy using cisplatin and new drugs has recently been approved and demonstrated to be effective in the improvement of patient survival (5, 6). However, not all patients can receive survival benefit by adjuvant chemotherapy. These results indicate the need for a more valuable surrogate serum marker for angiogenesis in order to select an appropriate neoadjuvant therapy including anti-angiogenic treatment.

Tenascin (Tn)-C is a large, secreted oligomeric extracellular matrix protein (ECM) that is expressed in the developing brain, cartilage, and mesenchyme and is re-expressed in tumors, wound healing and inflammation (7). Tn-C consists of several isoforms derived from alternative splicing of its mRNA (8). It has recently been demonstrated that the Tn-C high molecular weight isoform regulates angiogenesis, resulting in up-regulation of tumorigenesis of various cancers (8-14). Studies concerned with the relationship between Tn-C serum concentration and the clinical stage of the tumor for colon carcinoma, malignant melanoma and squamous cell carcinoma of the head and neck have been reported, but are few in number (15-17). However, a limited number of reports has focused on the involvement of Tn-C in lung cancer progression, particularly from the aspect of angiogenesis (18-20). For instance, Kusugawa et al. (18) demonstrated the expression of Tn-C and its degradation in human lung cancers. They investigated the correlation between the amount of protein and mRNA expression of Tn-C in surgically resected...
tumor specimens and the histopathological stage, but did not evaluate its role in angiogenesis in lung cancer. Moreover, this method is not capable of predicting intratumoral angiogenic activity prior to surgery. To the best of our knowledge, there are no studies reporting on the association between serum Tn-C concentration and angiogenesis in the primary tumor of NSCLC.

In this study, we revealed a positive correlation for preoperative concentration of serum Tn-C in reference to intratumoral angiogenesis and serum levels of other angiogenic factors (VEGF and OPN). Interestingly, no correlation was demonstrated between Tn-C serum level and the clinical stage. We also clarified that preoperative serum Tn-C is a valuable marker for the prediction not only of intratumoral angiogenesis, but also patient survival.

**Patients and Methods**

**Patients.** A total of 63 patients with primary NSCLC (registered at Juntendo University Hospital, Japan, from 1992 to 2002) were evaluated in this study, including 43 males and 20 females with a mean age of 65±10 years (Table I). For all patients, the diagnosis of lung cancer was confirmed by histological examinations of either biopsy or cytological specimens. Staging was determined based on the new international staging system. The procedure included chest radiography, computer tomography (CT) scans of the chest and abdomen, magnetic resonance imaging of the brain and bone scanning. Adenocarcinoma was diagnosed in 43 patients, squamous cell carcinoma in 15 patients, adenosquamous cell carcinoma in 2 patients, large cell carcinoma in 1 patient, carcinosarcoma in 1 patient and pleomorphic carcinoma in 1 patient. Eighteen patients were classified as Stage IIA, 26 patients as Stage IIB, 8 patients as Stage IIIA and 11 patients as Stage IIIB. Forty-nine healthy volunteers, with mean age of 49 ± 2 served as controls.

**Blood samples.** Blood samples were drawn preoperatively, centrifuged at 2500 rpm for 15 min and aliquots were frozen at −80°C until analysis. The protocol was approved by the Committee for Medical Ethics of Juntendo University, School of Medicine, and informed consent was obtained from all subjects enrolled in this study.

**Measurement of serum Tn-C and VEGF concentrations.** Serum concentrations of Tn-C and OPN were measured with commercially available sandwich ELISA kits (IBL, Gumma, Japan), according to the manufacturer's instructions. Serum samples were analyzed for VEGF with commercially available sandwich ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA). The limits of sensitivity were 0.38 ng/ml for Tn-C, 0.6 ng/ml for OPN and 9 pg/ml for VEGF.

**Measurement of serum CEA and SLX.** Serum concentrations of carcinoembryonic antigen (CEA) and sialyl lewis-X (SLX) were determined using the chemiluminescent immunoassay (CLIA) for the former and radioimmunoassay (RIA) for the latter, according to the manufacturers' instructions, respectively (CEA: Abbot Laboratories, North Chicago, IL, USA; SLX, Otsuka Pharmacoceutical Co., Tokyo, Japan).

**Immunohistochemical staining and microvessel counting.** Microvessels within the tumor tissue and Tn-C expression were evaluated by immunohistochemical analysis using the streptavidin-biotin method (8, 21). Briefly, paraffin-embedded tumor tissue samples were sectioned at the thickness of 3 μm and deparaffinized in xylene. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase in methanol. The sections were treated by autolaving for 15 min at 120°C in 10 mM citrate buffer, pH 6.0, to retrieve the antigen. These sections were incubated with normal horse serum for 15 min at room temperature, and then reacted with anti-CD34 monoclonal antibody (QBEnd 10, Medical Biological Laboratories, Nagoya, Japan) for 1 h at room temperature. After washing with PBS, the
sections were treated with biotin-labelled horse anti-mouse immunoglobulin for 20 min. The sections were washed with PBS and streptavidin-peroxidase complex was added. Color development was performed with 3-3’ diaminobenzidine (Sigma, St. Louis, MI, USA) in 0.03% hydrogen peroxide, and counterstained with hematoxylin. Tumor sections were microscopically scanned and the areas with the highest microvessel density (MVD) were identified. Microvessel counts were performed in five areas with the highest vascular density at 100 magnification and expressed as a mean value. For double staining of CD34 and Tn-C, sections stained with anti-CD34 antibody were washed again with TBS (0.1M Tris-buffered saline, pH 8.2 containing 0.05% Tween), followed by blocking with normal horse serum for 10 min at room temperature. These sections were incubated with anti-human Tn-C monoclonal antibody (4C8MC, IBL) for 1h at room temperature. After washing with TBS, the sections were treated with biotin-labelled horse anti-mouse immunoglobulin for 20 min. The sections were washed with TBS and streptavidin-alkaline phosphatase complex was added. Color development was performed using Vector Blue alkaline phosphatase substrate kit III (Vector Laboratories, Burlingame, CA, USA) in 0.03% hydrogen peroxide.

In vitro cell migration assay. In vitro cell proliferation assay was performed with the Boyden chamber method modified, as previously described (22, 23). Briefly, 1 x 10⁵ human umbilical endothelial cells (HUVEC) were plated onto the inserts with 8 μm pore membrane (Becton Dickinson, Franklin, NJ, USA). The reverse sides of the membranes were coated with either recombinant Tn-C (100 μg/ml), VEGF (10 ng/ml) or bovine serum albumin (BSA; 100 μg/ml). The lower chambers of the 24-well plate were filled with Opti-MEM containing 0.1% BSA. HUVEC were allowed to incubate at 37°C. After 6-h incubation, the cells were fixed with 10% neutralized buffered formalin for 15 min and were stained with 0.3% crystal violet for 10 min. After three washings with Milli-Q water, the remaining cells on the surface of the membrane were wiped clean with cotton swabs. Stained cells were calculated by low power magnification.

Statistical analysis. All data are presented as mean±SD. The relationship between serum Tn-C and various clinicopathological parameters were analyzed using the Mann-Whitney U-test with the exception of c-stage. The Kruskal-Wallis method was used to compare serum Tn-C levels with c-stage. Pearson’s correlation by rank was used to evaluate the correlations between serum Tn-C levels and microvessel density (MVD), VEGF, osteopontin (OPN) or various other tumor markers. Overall patient survival was analyzed by Kaplan-Meier method using the log-rank test. The criterion of statistical significance was p<0.05. Statview version 5.0 (Abacus Concepts) was used for all analyses.

Figure 2. The correlation between intratumoral microvessel density (MVD) (A), serum VEGF level (B) and serum OPN level (C) with serum Tn-C level in patients with NSCLC. A significant correlation was revealed between serum Tn-C level and intratumoral MVD (A: n=62, r=0.262, p=0.0469), serum VEGF level (B: n=62, r=0.457, P=0.0002) and serum OPN level (C: n=62, r=0.439, p=0.0004).
Results

Comparison between serum Tn-C levels in patients with NSCLC and those of healthy volunteers. We first compared serum Tn-C levels of patients with NSCLC with those of the healthy volunteers. As shown in Figure 1, the median concentration of serum Tn-C in NSCLC patients who underwent surgery was slightly higher than that of the healthy volunteers, although this difference did not reach statistical significance (p=0.1189).

Relationship between serum Tn-C level and various clinical factors in patients with NSCLC. The relationship between clinicopathological findings and serum Tn-C concentrations in all patients with NSCLC is shown in Table I. There was no difference in serum Tn-C levels among gender and histological type. Interestingly, there was no significant correlation between serum Tn-C level and the clinical-stage classification.

Correlation between serum Tn-C level and microvessel density (MVD), VEGF, OPN levels and various tumor markers in the tumor tissue of NSCLC. We assessed the relationship between serum Tn-C level and intratumoral MVD by immunohistochemical analysis. There was a weak correlation between serum Tn-C level and intratumoral MVD (Figure 2A, r=0.262, p=0.0469). The correlation between serum Tn-C level and serum VEGF, OPN levels and various tumor markers (CEA and SLX) was also analyzed. As expected, there was a significant correlation between serum Tn-C and both VEGF (Figure 2B) and OPN level (Figure 2C) (r=0.457, p=0.0002, r=0.439, p=0.0004, respectively). In contrast, the serum Tn-C level did not correlate with either serum CEA (Figure 3A) or SLX level (Figure 3B) (r=0.095, p=0.463, r=0.054, p=0.676, respectively).

Correlation of serum Tn-C levels with overall survival of the patients with NSCLC. We analyzed the influence of serum Tn-C levels on overall survival according to the Kaplan-Meier method. A statistically significant difference in the average overall survival between patients with high Tn-C value (upper quartile group; ≥96 ng/ml, n=16) and patients with low Tn-C value (<96 ng/ml, n=47) was revealed (Figure 4, p=0.0141).
Expression of Tn-C in NSCLC with immunohistochemical staining. To investigate Tn-C expression in NSCLC tissues, immunohistochemical analysis was performed. Immunoreactivity of Tn-C was observed mainly in the stroma, but not in cancer cells. As shown in Figure 5, the majority of Tn-C was co-expressed with CD34-positive endothelial cells in the stroma, suggesting the involvement of Tn-C in angiogenesis.

In vitro HUVEC migration. As shown in Figure 6, HUVEC migrated toward the reverse side of the membrane coated with not only recombinant VEGF but also Tn-C to a greater degree than those coated with bovine serum albumin. Addition of Tn-C (100 μg/ml) into the upper chamber abrogated the Tn-C-mediated HUVEC migration (data not shown). These results suggest that Tn-C is directly involved in endothelial cell migration.

Discussion

Tn-C has been reported to regulate angiogenesis in tumor through its role in the regulation of VEGF expression (24). Moreover, both Tn-C and VEGF are expressed during the peak of angiogenesis, and disappear when angiogenesis ceases (15). In fact, several researchers have demonstrated that Tn-C is involved in neoplastic angiogenesis in various cancers including breast cancers, astrocytoma, and oral and pharyngeal squamous cell carcinoma (10-14, 25). However, no reports have ever investigated its clinical role in the development of lung cancer, particularly from the point of view of angiogenesis. In this study, we determined the serum concentration of Tn-C, several angiogenic factors and tumor markers in patients with operable NSCLC. The relationship between serum Tn-C levels and the levels of angiogenic factors (e.g. VEGF and OPN) were revealed to be significantly correlated. Serum concentrations of Tn-C correlated with intratumoral MVD, which represents neoplastic angiogenic activity. Moreover, the immunohistochemical analysis revealed that Tn-C is co-localized with CD34-positive endothelial cells. These results, together with the in vitro finding that HUVEC strongly migrated toward Tn-C, suggest that Tn-C may play an important role in angiogenesis in NSCLC. To the best of our knowledge, this may be the first report in which the serum level of Tn-C was demonstrated to be a useful predictive marker of intratumoral angiogenesis in NSCLC.

Compared to immunohistochemical analysis, the measurement of serum Tn-C using ELISA can be easily performed. Moreover, the blood sample can be obtained prior to surgery. If neoplastic angiogenic activity of the tumor and patient prognosis could be predicted prior to surgery, patients who should undergo neoadjuvant chemotherapy could be selected. However, only a limited number of studies have focused on the clinical implication of circulating Tn-C in serum (15-17, 26, 27). For instance, Burchardt et al. (16) have recently revealed that a significant increase in Tn-C serum level was observed in patients with stage IV melanoma in comparison to healthy controls. Pauli et al. (17) reported on elevated levels of serum Tn-C in patients with higher tumor stages or recurrent squamous cell carcinoma of the head and neck. In colorectal carcinoma, the preoperative levels of serum Tn-C were demonstrated to reflect on the total tumor burden and correlate with metastatic disease activity (15). These results suggest that serum Tn-C may be a potential tumor marker in certain cancers. However, there have been no studies defining the association of circulating Tn-C with either intratumoral angiogenesis or prognosis of NSCLC. In this study, we first revealed a positive correlation of Tn-C serum levels with not only intratumoral MVD, but also with other angiogenic factors, VEGF and OPN. In contrast, we could not demonstrate any relationship between Tn-C serum levels and either CEA, SLX or clinical-stage. Collectively, the Tn-C serum level may not reflect on the total tumor burden in NSCLC, and thus it may not be categorized as a tumor marker. This idea is supported by our immunohistochemical findings that intense and diffuse Tn-C staining was observed in the stroma but not in cancer cells of NSCLC.

Many researchers have investigated whether Tn-C expression in tissue specimens could be a potential prognostic marker in various cancers (15, 25, 28-36). However, the result still remains controversial. In some cancers, high Tn-C expression in tumor has been reported to correlate with poor prognosis (28, 29, 31, 32). In contrast, high Tn-C expression has been associated with better prognosis in some other cancers (30, 34, 37). Recently, Cai et al. (19) reported that degradation of Tn-C is associated with tumor recurrence in early stage NSCLC. This appears to be the first paper describing the relationship between Tn-C expression and clinical outcome in lung cancer. However, they did not mention the merit of serum Tn-C level on patient survival. In this study, we first revealed that the overall survival of patients with high Tn-C serum levels (upper quartile group) was significantly shorter than that of patients with low serum Tn-C levels. Since this difference is subtle, further large-scale studies are necessary to confirm the result.

In conclusion, we revealed that the preoperative concentration of serum Tn-C is a valuable marker for the prediction of intratumoral angiogenesis and prognosis in NSCLC. Determination of serum Tn-C may be of value when neoadjuvant therapy, including anti-angiogenic therapy, is considered for patients with NSCLC.
References


Figure 5. Histological finding of Tn-C and CD34 expressions in the tumor tissue specimen of adenocarcinoma with immunohistochemical analysis. Note that the majority of Tn-C was expressed in the stroma of the tumor and co-localized with CD34-positive endothelial cells (x400). Tn-C is stained blue, while CD34 is stained brown in color. Interestingly, there was no Tn-C-positive staining in the cancer cells.

Figure 6. In vitro HUVEC migration toward recombinant Tn-C. HUVEC (1 x 10^5) were placed in the upper chamber and were allowed to migrate toward the reverse side of the membrane coated with Tn-C, VEGF or BSA. HUVEC migrated toward recombinant Tn-C or VEGF to a greater degree than BSA. Data are expressed as mean ± S.D. of migrated cell number of five different microscopic fields observed, magnification x100. *p=0.019 vs. BSA **p=0.0002 vs. BSA.

Received September 24, 2004
Accepted December 20, 2004

Ishiwata et al: Serum Tenasin in Lung Cancer