Tumor-derived Tenascin-C Promotes the Epithelial–Mesenchymal Transition in Colorectal Cancer Cells

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Abstract. Background: Tenascin-C (TNC) is an extracellular matrix glycoprotein, usually derived from myofibroblasts in the cancer microenvironment. Recently, however, the significance of tumor-derived TNC in initiation of cancer metastasis was disclosed. We investigated the clinical significance of cancer-derived TNC in colorectal cancer (CRC) cases. Materials and Methods: TNC expression in 170 cases of CRC was analyzed by quantitative real-time polymerase chain reaction (PCR). In addition, gene expression arrays using purely-separated cancer tissues of another 86 cases was performed and the functional implications of cancer-specific TNC were investigated. Results: The expression of TNC mRNA was significantly higher in CRC tissues than in the corresponding normal tissues. Cancer cell-specific TNC expression was a significant prognostic factor in CRC cases. Moreover, cancer cell-derived TNC was associated with the epithelial–mesenchymal transition (EMT) signature. Conclusion: Cancer cell-derived TNC promotes cancer invasiveness via EMT regulation, and not cancer tissue TNC but cancer cell-specific TNC is a novel indicator of poor prognosis.

In Japan, mortality and morbidity associated with colorectal cancer (CRC) are increasing exponentially, and CRC is now considered to be a critical national health problem. Thus, the identification of factors regulating the invasiveness and metastasis of CRC would contribute to the improvement of clinical outcomes from treatment.

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In CRC, myofibroblasts in the tumor stroma of the invasive front have been shown to secrete TNC and stimulate cancer cell invasion (14). Some reports have shown that stromal expression or plasma expression of TNC is a useful biomarker (15). However, the prognostic significance of TNC in patients with CRC is controversial (16). Moreover, these studies also investigated TNC expression in stromal tissues. In the current study, we investigated the clinical significance of cancer-derived TNC in patients with CRC.

Materials and Methods

Patients and sample collection. All patients provided written informed consent before participation. A total of 256 CRC samples were obtained during surgery. Out of these samples, 170 were used in bulk (Set 1) and 86 were used as pure cancer tissues separated by laser microdissection (Set 2). All patients underwent resection of the primary tumor at Kyushu University Hospital at Beppu and affiliated hospitals between 1992 and 2007. All patients had a clear histological diagnosis of CRC and were closely followed-up every three months. The follow-up periods ranged from 0.1 to 12.3 years, with a mean of 3.8 years for Set 1 and from 0.1 months to 3.2 years, with a mean of 2.1 years for Set 2. Resected cancer tissues were immediately removed, stored in RNAlater (Ambion, Carlsbad, CA, USA) or embedded in Tissue-Tek OCT (Optimum Cutting Temperature) medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen, and kept at –80˚C until RNA extraction. Frozen tissue specimens were homogenized in guanidium thiocyanate, and total RNA was obtained by ultra-centrifugation through a cesium chloride cushion. cDNA for reverse transcription polymerase chain reaction (PCR) was synthesized from 8.0 μg of total RNA with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Clinicopathological factors and clinical stages were classified using TNM systems of classification (17). All sample data, including age, gender, histology, tumor depth, lymph node metastasis, lymphatic invasion, vascular invasion, liver metastasis, and postoperative recurrence in liver, were obtained from the clinical and pathological records.

Laser microdissection. Tissue samples were microdissected using an LMD6000 laser microdissection system (Leica Laser Microdissection System; Leica Microsystems, Wetzlar, Germany) as previously described (18). For laser microdissection, 5-μm-thick frozen sections were fixed in 70% ethanol for 30 s, stained with hematoxylin and eosin, and de-hydrated as follows: 5 s each in 70%, 95%, and 100% ethanol. Sections were air-dried and then microdissected with the LMD system. Target cells were excised, with at least 100 cells per section, and bound to the transfer film. Total RNA was then extracted.

Evaluation of gene expression levels in clinical samples. For quantitative real-time reverse transcription (qRT)-PCR, TNC (NM_002160.2) primer sequences were as follows: sense, 5’-CGGGGCTATAGACACCGAAGT-3’ and antisense, 5’-AACATTTAGTGTCATCATATTGGCAGGTTA-3’. To normalize for RNA concentration, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. The sequences of the GAPDH primers were as follows: sense, 5’-TTGGTATCGTGGAAGGAC-3’ and antisense, 5’-TGTCATCATATTGGCAGGTT-3’. The amplification protocol included an initial denaturation step at 95˚C for 10 min, followed by 45 cycles of 95˚C for 10 s and 60˚C for 30 s. qRT-PCR was performed in a LightCycler 480 instrument (Roche Applied Science, Basel, Switzerland) using the LightCycler 480 Probes Master kit (Roche Applied Science). All concentrations were calculated relative to the concentration of cDNA using Human Universal Reference Total RNA (Clontech, Palo Alto, CA, USA). The concentration of TNC was then divided by the concentration of the endogenous reference (GAPDH) to obtain normalized expression values.

Gene expression microarray. We used the commercially available Human Whole Genome Oligo DNA Microarray Kit (Agilent Technologies, Santa Clara, CA, USA). A list of genes on this cDNA microarray is available from http://www.chem.agilent.com. Cyanine (Cy)-labeled cRNA was prepared using T7 linear amplification as described in the Agilent Low RNA Input Fluorescent Linear Amplification Kit Manual (Agilent Technologies). Labeled cRNA was fragmented and hybridized to an oligonucleotide microarray (Whole Human Genome 4×44K Agilent G4112F). Fluorescence intensities were determined with an Agilent DNA Microarray Scanner and were analyzed using the G2567AA Feature Extraction Software Version A.7.5.1 (Agilent Technologies), which used the locally weighted linear regression curve fit (LOWESS) normalization method (19). This microarray study was conducted following the minimum information about a microarray experiment (MIAME) guidelines issued by the Microarray Gene Expression Data group (20). Gene expression arrays have been deposited at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database with accession code GSE21815.
**Cell lines.** The human CRC cell lines DLD-1 and LoVo were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 (for DLD-1) or Ham’s F12 (for LoVo) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. All cells were cultured in a humidified 5% CO2 incubator at 37˚C.

**TNC RNA interference.** TNC-specific siRNA (Custom siSET) was purchased from Cosmo Bio (Tokyo, Japan). The siRNA oligomer was diluted with Opti-MEM I medium without serum (Invitrogen). The diluted siRNA oligomer was then mixed with diluted Lipofectamine RNAiMAX (Invitrogen) and incubated for 15 min at room temperature to allow for siRNA–lipofectamine RNAiMAX complexes to form. Diluted logarithmic growth-phase DLD-1 and LoVo cells without antibiotics were seeded at 2×10^5 or 5×10^4 cells per well in a final volume of 2 ml or 500 μl, respectively, in 6- or 24-well flat-bottom microtiter plates, respectively. The cells were then incubated in a humidified atmosphere (37°C and 5% CO2) for 24 h. The assay was performed after a 24-h incubation.

**Cell proliferation analysis.** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Applied Science) was used to evaluate cell proliferation. After incubation, 10 μl of MTT labeling reagent (at a final concentration of 0.5 mg/ml) was added to each well, and the plate was incubated for 4 h in a humidified atmosphere.

Solubilization solution (100 μl) was added to each well, and the plate was incubated overnight in a humidified atmosphere. After confirming that the purple formazan crystals were completely solubilized, the absorbance of each well was measured by a Model 550 series microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 570 nm corrected to 655 nm. The assay was performed using six replicates.

**Gene set enrichment analysis (GSEA) of CRC expression.** Gene expression profiles of 86 CRC cancer-specific samples were measured by GSEA. For GSEA, TNC expression was treated as a numeric variable. We applied a continuous-type class (CLS) file of the TNC profile to phenotype labels in GSEA. The metric for ranking genes in GSEA was set as “Pearson,” and the other parameters were set to their default values.

**Statistical analysis.** Data from RT-PCR analyses and in vitro transfected cell assays were analyzed using the JMP 5 software (JMP, Cary, NC, USA). Overall survival rates were calculated actuarially according to the Kaplan–Meier method and were measured from the day of surgery. Differences between groups were estimated using the χ^2 test, Student’s t-test, repeated-measures ANOVA, and log-rank test. A probability level of 0.05 was chosen for statistical significance.

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**Table I. Tenascin-C (TNC) mRNA expression and clinicopathological factors in 170 cases of colorectal cancer.**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Low expression (n=85)</th>
<th>High expression (n=85)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt;65 years</td>
<td>30 35.29</td>
<td>35 41.18</td>
<td>0.43</td>
</tr>
<tr>
<td>Age ≥66 years</td>
<td>55 64.71</td>
<td>50 58.82</td>
<td>0.642</td>
</tr>
<tr>
<td>Sex Male</td>
<td>50 58.29</td>
<td>47 55.29</td>
<td>0.387</td>
</tr>
<tr>
<td>Sex Female</td>
<td>35 41.18</td>
<td>38 44.71</td>
<td></td>
</tr>
<tr>
<td>Histological grade Well/moderate</td>
<td>80 94.12</td>
<td>77 90.59</td>
<td>0.833</td>
</tr>
<tr>
<td>Histological grade Other</td>
<td>5 5.88</td>
<td>8 9.41</td>
<td></td>
</tr>
<tr>
<td>Tumor size &lt;30 mm</td>
<td>56 68.29</td>
<td>65 83.33</td>
<td>0.027*</td>
</tr>
<tr>
<td>Tumor size ≥30 mm</td>
<td>26 31.71</td>
<td>13 19.67</td>
<td></td>
</tr>
<tr>
<td>Serosal invasion Absent</td>
<td>47 55.29</td>
<td>18 21.81</td>
<td>0.001*</td>
</tr>
<tr>
<td>Serosal invasion Present</td>
<td>38 44.71</td>
<td>67 78.82</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis N0</td>
<td>56 65.88</td>
<td>36 42.86</td>
<td>0.003*</td>
</tr>
<tr>
<td>Lymph node metastasis N1–2</td>
<td>29 34.12</td>
<td>48 57.14</td>
<td></td>
</tr>
<tr>
<td>Lymphatic invasion Absent</td>
<td>67 78.82</td>
<td>37 44.05</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Lymphatic invasion Present</td>
<td>18 21.18</td>
<td>47 55.95</td>
<td></td>
</tr>
<tr>
<td>Venous invasion Absent</td>
<td>75 88.24</td>
<td>62 73.81</td>
<td>0.017*</td>
</tr>
<tr>
<td>Venous invasion Present</td>
<td>10 11.76</td>
<td>22 26.19</td>
<td></td>
</tr>
<tr>
<td>Liver metastasis Absent</td>
<td>75 89.41</td>
<td>76 89.41</td>
<td>0.417</td>
</tr>
<tr>
<td>Liver metastasis Present</td>
<td>6 7.06</td>
<td>9 10.59</td>
<td></td>
</tr>
<tr>
<td>Peritoneal dissemination Absent</td>
<td>85 100</td>
<td>79 92.94</td>
<td>0.013*</td>
</tr>
<tr>
<td>Peritoneal dissemination Present</td>
<td>0 0</td>
<td>6 7.06</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis Absent</td>
<td>84 98.82</td>
<td>82 96.47</td>
<td>0.312</td>
</tr>
<tr>
<td>Distant metastasis Present</td>
<td>1 1.18</td>
<td>3 3.52</td>
<td></td>
</tr>
<tr>
<td>UICC stage 0, I, II</td>
<td>55 64.71</td>
<td>35 41.18</td>
<td>0.002*</td>
</tr>
<tr>
<td>UICC stage III, IV</td>
<td>30 35.29</td>
<td>50 58.82</td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant. Well, Well-differentiated tubular adenocarcinoma; moderate, moderately-differentiated tubular adenocarcinoma; UICC, Union for International Cancer Control.
Results

Expression of TNC in clinical tissue specimens and clinicopathological characteristics. TNC mRNA expression in the bulk 170 tumor tissues, containing tumor and stroma, and corresponding normal tissues (Set 1), was examined by qRT-PCR to investigate the clinical importance of TNC expression in CRC. TNC mRNA levels in cancerous tissues were significantly higher than those in non-cancerous tissues (p<0.0001; Figure 1). We divided the 170 patients with CRC into a high-TNC expression group (n=85) and a low-TNC expression group (n=85), classified as having expression levels higher or lower than the median value, respectively.

Clinicopathological factors were compared between the high- and low-TNC mRNA expression groups (Table I). The high-TNC expression group (n=85) had larger tumor sizes (p=0.027) than the low-TNC mRNA expression group (n=85). Moreover, increases in factors such as tumor depth (p=0.001), lymph node metastasis (p=0.003), lymphatic invasion (p<0.0001), venous invasion (p=0.017), and peritoneal dissemination (p=0.013) were recorded in the high-TNC expression group, suggesting that TNC is involved in tumor invasiveness.

Relationship between tumor-specific TNC expression and prognosis. Next, we analyzed the prognoses of Set 1 cases. For overall survival, patients with high TNC mRNA expression had a poorer prognosis than those with low mRNA expression, but this difference was not significant (p=0.0648; Figure 2A). However, a significant difference was observed in the disease-free survival rate. The high-TNC expression group (n=74) had a significantly higher recurrence rate than the low-TNC expression group (p=0.0429; Figure 2B).

According to an online prognosis prediction algorithm, PrognoScan (21), cases with high TNC mRNA expression had significantly poorer disease-free survival [GSE12945 (22), n=51, p=0.006125; GSE17536, n=177, p=0.017016] and overall survival (GSE17536, n=177, P=0.015056).

Furthermore, we examined the overall survival of Set 2 cases to elucidate the clinical consequences of cancer-specific TNC mRNA expression, as evaluated by gene

![Figure 2. A: Kaplan–Meier analysis of overall survival for 170 patients with colorectal cancer (CRC) according to TNC mRNA expression (Set 1; bulk sample). B: Kaplan–Meier analysis of disease-free survival for 149 patients with CRC according to TNC mRNA expression (Set 1; bulk sample). C: Kaplan–Meier analysis of overall survival for 86 patients with CRC according to TNC mRNA expression (Set 2; purified, separated cancer cell sample).]
expression arrays. The high-TNC expression group had significantly poorer prognoses than the low-TNC expression group ($p=0.0429$; Figure 2C). These data suggest that cancer-specific TNC expression has a critical function in cancer progression.

In vitro assessment of TNC expression knockdown. Using qRT-PCR, we confirmed that TNC expression in cells transfected with TNC siRNA was significantly lower than that in untransfected cells (parent) and in cells transfected with negative control siRNA (LoVo, $p<0.0001$; DLD-1,
To explain the cancer-promoting effects of TNC in CRC cells, a proliferation assay was carried out in cells transfected with TNC siRNA and those transfected with negative-control siRNA (both LoVo and DLD-1 cell lines). TNC siRNA potently suppressed the proliferation of both of these CRC cell lines (LoVo, \( p < 0.05 \); DLD-1, \( p < 0.05 \); Figure 3B).

**Tumor-derived TNC promoted EMT and invasiveness.** In order to determine the mechanism through which tumor-derived TNC promotes CRC progression, we performed GSEA (23) using the data from gene expression arrays of Set 2 cases. The results of GSEA showed that gene sets related to EMT, TGF-β signaling, and cancer invasiveness were significantly enriched, with a false-discovery rate of less than 0.05 (Figure 4).

**Discussion**

Many previous studies have demonstrated that TNC is produced by myofibroblasts in the tumor microenvironment and that the expression of TNC in the cancer stroma or plasma predicted patient prognosis. Recently, however, Oskarsson et al. reported that cancer cell-derived TNC promotes the survival and outgrowth of pulmonary micrometastases in breast cancer (13). Their data also showed that cancer cells in micrometastases act as their own main source of TNC until the tumor stroma takes over as a source of TNC. Moreover, TNC has been shown to enhance the expression of the stem cell signaling component musashi homolog-1 (MSI1) and leucine-rich repeat-containing G protein-coupled receptor-5 (LGR5) without affecting the expression of transcriptional enforcers of the stem cell phenotype and pluripotency, namely nanog homeobox (NANOG), POU class 5 homeobox 1 (POU5F1, also known as OCT4), and sex determining region Y (SRY)-box 2 (SOX2).

In the current study, we investigated the clinical significance of cancer cell-derived TNC. Our data did not suggest that TNC supports the fitness of cancer-initiating stem cells. However, GSEA using cancer cell-specific expression profiles revealed the enrichment of gene sets...
associated with EMT, TGF-β signaling, and cancer invasion. These results indicate that cancer-derived TNC acts as a regulator of the EMT and promotes cancer invasiveness and disease relapse in patients with CRC. In fact, tumor-specific TNC expression was a robust indicator of disease prognosis, and knockdown of TNC in CRC cells dramatically suppressed cancer proliferation.

Tumor and metastasis initiation are tightly-regulated by suppressive organized epithelial structures and microenvironments. Cancer cells, which have the ability to evade suppressive microenvironments, cause tumor expansion (24). Cancer cell-derived TNC seems to act in tumor initiation and micrometastases in such suppressive environments until the tumor stroma is organized. In addition, some reports have indicated that therapies targeting metastasis-initiating cells can be efficacious (13, 25). Thus, the therapeutic targeting of TNC may provide a strategy for eliminating tumor cells that cause disease metastasis and relapse in patients with CRC.

Conflicts of Interest

None.

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