Tumor-Associated Antigen Recognized by the 22-1-1 Monoclonal Antibody Encourages Colorectal Cancer Progression under the Scanty CD8+ T Cells

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Abstract

Purpose: The receptor-binding cancer antigen expressed on SiSo cells (RCAS1) is a novel tumor-associated antigen. Although evidence suggests that RCAS1 suppresses immunity by inducing tumor-infiltrating lymphocyte (TIL) apoptosis, RCAS1 function in humans is controversial. RCAS1 overexpression leads to the generation of the Tn glycan antigen (α-N-acetyl-D-galactosamine, GalNAc) recognized by the 22-1-1 monoclonal antibody. The objective of this study is to examine Tn glycan antigen function in colorectal cancer and to determine its relationship to CD8+ T cells and prognosis.

Experimental Design: Immunohistochemical analyses examined Tn expression in tumor cells and CD8 on TILs in 146 surgically resected colorectal cancer.

Results: Of 146 samples, 68 tumors (47%) were Tn+; 72 tumors (49%) were CD8+. Using Cox multivariate analysis and the Kaplan-Meier method, Tn and CD8 positivity were determined to be mutually independent prognostic factors (P = 0.0266 and 0.0210, respectively). Tn+ patients with CD8+ TILs exhibited better survival than Tn+/CD8− patients (P = 0.0129). For CD8− patients, Tn positivity was associated with decreased survival from that seen in Tn− patients (P = 0.0097), suggesting that Tn exerts a function independent of CD8+ T cells in tumor progression. In all patients and cases with synchronous liver metastases (n = 29), the Tn+/CD8− survival rate was significantly lower than that seen for other groups (P = 0.0001 and 0.0063, respectively). The average number of liver metastases in Tn+/CD8− cases also increased (mean, 8.2 tumors; P = 0.0032). Multivariate analysis identified Tn+/CD8− status and Dukes' staging as independent prognostic factors (P = 0.0016 and 0.0001, respectively).

Conclusions: Tn may encourage invasion and innidiation through a mechanism independent of CD8+ T cells. Thus, Tn+/CD8− status is a risk factor for multiple liver metastases development and an independent negative prognostic factor for colorectal cancer.

The prognosis of malignant human colorectal cancer is relatively good. In the carcinogenesis of colorectal cancer, a multistep process, a portion of these tumors progress to advanced malignancy and eventually metastases (1–3). Previous reports have suggested that immune reactions of the host diminish the aggressiveness of colorectal cancer cells; thus, immunosuppressive environments are required for cancer cells to survive in the body (4–7).

Initially, a novel tumor-associated antigen, receptor binding cancer antigen expressed on SiSo cells (RCAS1), was defined by the 22-1-1 monoclonal antibody (8). Immunohistochemistry revealed positive cell surface staining with 22-1-1 in a large number of different tumor tissues. In some tumor entities, 22-1-1 staining correlated with poor clinical prognosis (9, 10). The physiologic molecular function of RCAS1 in humans, however, remains controversial.

Tumor-infiltrating lymphocytes (TIL) are a manifestation of host immune responses against cancer cells (11). TILs in a variety of carcinoma types have been reported to be a positive prognostic factor (3, 12–14). The presence of CD8+ T cells in the TIL population seems important in the anticancer immune response. RCAS1 may act as a ligand for a putative receptor present on immune cells, such as natural killer cells and activated T cells, inducing apoptosis (15). TILs have been detected surrounding RCAS1-positive tumor cells in human oral squamous cell carcinomas (16). Nakamura et al. observed in human gastric carcinoma that the rate of TIL apoptosis was significantly higher in RCAS1-positive areas than in RCAS1-negative areas (17). Recently, Engelsberg et al. reported that RCAS1 modulates surface expression of a tumor-associated, normally cryptic O-linked glycan structure α-N-acetyl-D-galactosamine, GalNAc (Tn). The monoclonal antibody 22-1-1 recognizes RCAS1 through Tn glycan antigen, which is identical to the 22-1-1 epitope. These
studies also suggested that RCAS1 contributes indirectly to the antigenicity of tumor cells, raising doubts as to the immuno-suppressive activity of RCAS1 (18). The mean number of CD8+ TILs in RCAS1-positive tumors did not differ from that of CD8+ TILs in RCAS1-negative tumors in hepatocellular carcinoma (19). Immunohistochemistry analyzing the correlation of Tn antigen expression and TIL phenotype and apoptosis induction may help to determine prognosis more accurately.

To determine if Tn antigen is independent of CD8+ T cells and to establish new prognostic factors for patients with colorectal cancer, we immunohistochemically investigated the correlation of Tn antigen expression and CD8+ T-cell tumor infiltration in large number patients with colorectal cancer.

Materials and Methods

Patients and specimens. Tumor specimens were obtained from 146 patients with colorectal cancer. These patients (95 males and 51 females; mean age, 63.5 years) underwent surgical resection at the Department of Surgery of Hokkaido Gastroenterology Hospital during a period between 1995 and 1997. All cases had follow-up data available for at least 7 years following resection. No cases received radiation or chemotherapy before operation. To stage individual tumors, we adopted Duke's Classification: A, cancer invasion was confined to the submucosa or the muscularis propria without metastasis (n = 52); B, the cancer had invaded into the subserosa or adventitia without metastasis (n = 34); C, lymph node metastases were present (n = 27); and D, tumor existed in conjunction with simultaneous hematogenous metastases (n = 33). In the Dukes' D patients, 29 cases (88% of Dukes' D patients) exhibited synchronous liver metastases without any other metastasis to other locations, including the lung and peritoneum. Liver metastases were diagnosed either by histopathologic examination of metastatic foci or by computed tomography. Liver resection was done for patients with less than three metastatic liver tumors; nine patients received hepatectomies.

Immunohistochemistry. Surgical specimens were fixed in 10% formalin and embedded in paraffin for sectioning at a 4-μm thickness. Immunohistochemical reactions were done using the streptavidin/biotin/ peroxidase method. Sections were deparaffinized in xylene, washed in PBS (pH 7.4), and rehydrated using a graded ethanol series. Endogenous peroxidase activity was inhibited by a 10-minute incubation with 3% hydrogen peroxide in methanol. Following washing with PBS, specimens were blocked in 10% normal goat serum (Histofine SAB-PO kit, Nichirei Corp., Tokyo, Japan) for 5 minutes. Samples were then incubated overnight at 4°C with one of the following mouse monoclonal primary antibodies: anti-human 22-1-1 (clone 22/1-1, DAKO, Glostrup, Denmark; diluted at 1:100), or anti-granzyme B (clone GrB-7; Kamiya Biomedical Co., Seattle, WA; diluted at 1:40). After washing in PBS, a 30-minute incubation at room temperature with biotinylated goat anti-mouse immunoglobulin (Histofine SAB-PO kit, Nichirei) identified regions of antibody staining. After extensive washing in PBS, immunohistochemical reactions were developed using freshly prepared 3,3'-diaminobenzidine tetrahydrochloride (Histofine SAB-PO kit, Nichirei). Slides were counterstained in hematoxylin and mounted on coverslips in systemic mounting medium. Normal adenoid tissue was used as a positive control for CD8 staining, whereas uterine adenocarcinoma tissue was used for 22-1-1. As a negative control, purified nonimmune mouse sera were substituted in place of specific primary antibody.

Evaluation and classification of Tn and CD8+ T cells. The degree of Tn immunoreactivity was classified into two categories as previously reported in colorectal cancer (20). Tn+ cases exhibited <50% of tumor cells with positive immunoreactivity, and in Tn+, >50% of tumor cells expressed Tn. Scoring of Tn immunoreactivity was based on an examination of 10 high-power (×400) microscopic fields, each containing >1,000 cells.

Immunohistochemistry and evaluation of CD8+ T cells were done as described by Naito et al. (21). The number of immunoreactive lymphocytes was quantified within a ×200 microscopic field (0.933 mm²). The three areas with the most abundant infiltrating cells were selected for quantitation; average numbers of CD8+ T cells of ≤17 and >18 were scored as CD8− and CD8+, respectively. The number of CD8+ T cells was counted both in the mesenchymal stroma and within the cancer cell nest.

We classified all patients into four groups for analysis according to Tn and CD8 positivity: patients with tumors classified as Tn+ and CD8+ were described as Tn+/CD8+. In this manner, the remaining three groups were designated as Tn−/CD8−, Tn+/CD8−, and Tn−/CD8−.

A sparsely granular pattern of granzyme B+ lymphocytes, within CD8+ samples, was considered positive. To estimate the immunoreactivity of granzyme B+ lymphocytes, an oil immersion lens was used.

Immunoreactivity of each section was reviewed by two independent investigators, blinded to the patients' clinical information. In cases of occasional discrepancies in interpretation, consensus was achieved after discussion with the aid of a multihed microscope.

Statistical analysis. To compare Tn+/CD8+ and Tn−/CD8− granzyme positivity, Mann-Whitney test was done. Kaplan-Meier methodology was used to generate survival curves. Survival differences were analyzed by the
log-rank test. Statistical analysis of the differences between the number of metastatic liver tumors was calculated using the Kruskal, Wallis, and Scheffe test. The influence of these variables on survival was assessed using Cox univariate and multivariate regression analyses. P < 0.05 were considered statistically significant. All analyses were done using Statview statistical software (version 5.0; SAS Institute, Inc., Cary, NC).

Results

Immunohistochemistry. Based on RACS1 expression, the 146 colorectal cancers examined in this study were divided into two groups. Sixty-eight tumors (47%) were classified as positive for Tn expression (Tn+). Of the 146 tumors, 72 of the 146 (49%) tumors contained significant infiltrating CD8+ T cells in the mesenchymal stroma or within the cancer cell nest. Of the 146 patients with colorectal cancer, 33 patients were classified as Tn+/CD8+ (Fig. 1A), 41 as Tn+/CD8- (Fig. 1B), 35 as Tn+/CD8+CD8+ (Fig. 1C), and 37 as Tn-/CD8+ (Fig. 1D).

Fig. 2. Kaplan-Meier analysis of overall survival relative to the amount of Tn expression in cancer cells (A) or the number of CD8+ T cells in the mesenchymal stroma and cancer cell nest (B) was examined in 146 colorectal cancer patients. Both values served as significant prognostic factors.

Fig. 3. Kaplan-Meier analysis of overall survival relative to the Tn/CD8 status of patients of all stages (n = 146; A), Dukes’ D stage patients (n = 33; B), or only those individuals with synchronous liver metastases in the absence of any other metastasis (n = 29; C). The prognosis of the Tn+/CD8+ group was significantly worse at each progressive stage.
From our examination of granzyme B+ expression within those cells positive for CD8, a collection of the CD8+ T cells within tumors were confirmed to have an activated, cytotoxic phenotype (22). In patients with CD8+, the average proportion of CD8+ T cells testing positive for granzyme B+ was 31.3% and 29.8% in the Tn+/CD8+ and Tn-/CD8+ groups, respectively. Statistical difference was not seen between two groups.

**Overall survival rates for Tn and CD8+ T cells.** Survival in patients exhibiting a high Tn expression pattern was significantly worse than that seen for patients with low-expression patterns (log-rank test, \( P = 0.0266 \); Fig. 2A). Overall survival rates were increased in colorectal cancer patients exhibiting TILs positive for CD8+ over those seen for patients negative for CD8+ T-cell infiltration (log-rank test, \( P = 0.0210 \); Fig. 2B).

**Kaplan-Meier survival analysis of Tn+/CD8+ and other patient populations.** Survival curves constructed according to the Kaplan-Meier method (Fig. 3A-C) show that the Tn+/CD8− group exhibited a significantly lower survival rate than the other three groups; the Tn-/CD8+ group displayed a significantly higher survival rate than the other three groups (log-rank test, \( P = 0.0001 \); Fig. 3A). In Tn+ patients, the presence of infiltrating CD8+ T cells (Tn+/CD8+ group) was associated with a significantly better survival rate than the absence (Tn-/CD8− group; log-rank test, \( P = 0.0129 \); Fig. 3A). Of the CD8+ patients, those with tumor cells positive for Tn (Tn+/CD8+ group) showed a significantly worse survival rate than patients with Tn-negative tumors (Tn-/CD8+ group; log-rank test, \( P = 0.0097 \); Fig. 3A). Among the Duke's D cancer patients (\( n = 33 \)), the Tn+/CD8− group exhibited a significantly lower survival rate compared with the other three groups (log-rank test, \( P = 0.0192 \); Fig. 3B). Of the 29 patients with synchronous liver metastases alone, the Tn+/CD8− phenotype (\( n = 10 \)) was associated with a significantly lower survival rate than that seen for the other groups (Tn+/CD8−, \( n = 8 \); Tn+/CD8+, \( n = 9 \); and Tn-/CD8−, \( n = 2 \); log-rank test, \( P = 0.0063 \); Fig. 3C).

**Number and surgical removal rate of synchronous liver metastases among Tn/CD8 status.** In patients with synchronous liver metastases (\( n = 29 \)), the number of liver tumors averaged 8.2, 3.9, 4.0, and 3.0 in the Tn+/CD8−, Tn+/CD8+, Tn-/CD8+, and Tn-/CD8− groups, respectively. The number of the liver tumors present in patients with Tn/CD8+/− tumors was significantly larger than that seen in the other groups (Kruskal, Wallis, and Scheffe test, \( P = 0.0032 \); Fig. 4). The surgical removal rate of liver lesions in the Tn/CD8+/−, Tn+/CD8+, Tn-/CD8+, and Tn-/CD8− groups was 10%, 38%, 44%, and 50%, respectively (Table 1).

**Univariate and multivariate survival analyses.** Cox univariate and multivariate regression analysis showed that both Tn and CD8+ positivity influenced prognosis independently (Table 2A). Univariate analysis identified a significant correlation among the histopathologic type, lymphatic invasion, venous invasion, Dukes' staging, and Tn/CD8 status with survival in 146 patients with colorectal cancer. Multivariate analysis of the same set of patients was used to analyze the influence of pathologic predictors and Tn/CD8 status on survival time using the Cox regression model. Tn+/CD8− status was determined to be an independent negative prognostic factor (hazard ratio, 2.724; \( P = 0.0016 \)). Dukes' staging also exhibited independent prognostic value with hazard ratio of 3.382 (\( P < 0.0001 \); Table 2B).

**Discussion**

Cell surface staining with monoclonal antibody 22-1-1 and the presence of CD8+ T cells are both reported to be prognostic factors in a variety of cancers (9, 10, 12, 23). In human colorectal cancer, the infiltration of CD8+ T cells into the cancer cell nest is a reliable positive prognostic indicator (21). Our results also showed an association of CD8+ T-cell infiltration with increased survival. RCAS1 is up-regulated both in serum and tumor tissues in the later stages of colorectal cancer (20). Some evidence suggests that RCAS1 may negatively affect the prognosis of human colorectal cancer (24). Our data also suggest that Tn expression significantly affected overall survival. Thus, our findings are in agreement with previous reports.

In contrast, Okada et al. reported that the proportion of apoptotic CD8+ TILs was significantly higher in RCAS1-positive colorectal cancer samples than in RCAS1-negative tumors (24). Their data suggest that the prognostic value of the presence of TILs, including CD8+ T cells, depends on RCAS1 expression.

**Table 1. Cases of surgical removal of synchronous liver metastases**

<table>
<thead>
<tr>
<th></th>
<th>Tn+/CD8−</th>
<th>Tn+/CD8+</th>
<th>Tn−/CD8−</th>
<th>Tn−/CD8+</th>
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<tbody>
<tr>
<td>Cases of synchronous liver metastases</td>
<td>10</td>
<td>8</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Cases of surgical removal of liver</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Rate of surgical removal of liver</td>
<td>10%</td>
<td>38%</td>
<td>44%</td>
<td>50%</td>
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</table>

NOTE: The rate of surgical removal of synchronous liver metastases in the Tn+/CD8− group was lower than in the other groups.
Using Cox regression multivariate analysis, however, our data indicated the independence of Tn and CD8+ T cells as prognostic indicators. There is significant difference between the prognosis of Tn+/CD8+ and Tn-/CD8- patients; if the primary effect of Tn on prognosis occurs through the induction of apoptosis of CD8+ T cells, such a significant difference in prognosis between these two groups would not likely be seen.

In conclusion, we could not find any evidence for a Tn function dependent on CD8+ T cells in colorectal cancer. Instead, we determined that Tn+/CD8+ status is a risk factor for unresectable multiple liver metastases and a negative independent prognostic factor in colorectal cancer. Collection and analysis of Tn/CD8 expression in colorectal cancer specimens may be useful to estimate the likelihood of unresectable multiple liver metastases and to decide an appropriate treatment strategy.

Cox multivariate analysis showed that Tn+/CD8- is an independent negative prognostic factor in colorectal cancer. The prognosis of the Tn+/CD8- group of patients with synchronous liver metastases, 88% of Dukes’ D patients, is extremely poor due to a higher number of metastatic liver tumors than seen in other groups, leading to a lower rate of liver resection. These data suggest that the Tn+/CD8- phenotype may be associated with synchronous multiple liver metastases, which cannot be treated by surgical resection. Tn antigens are linked to increased cell adhesion, invasion, and metastasis of cancer cells (25). In the absence of a host CD8+ T cell’s response, Tn antigens may encourage the metastatic phenotype observed in these patients.

In addition, the difference in prognosis between the Tn+/CD8+ and Tn-/CD8- groups, which occurs in the absence of a part of host immune response, suggests that expression of Tn affects prognosis independent of any CD8+ T cell’s suppressive ability. We do not provide any evidence that Tn expression contributes to evasion of the host CD8+ T cell’s response by colorectal cancer. However, our data cannot entirely eliminate the immunosuppression hypothesis of Tn. Because only CD8+ lymphocyte populations were examined in this study, we should give careful consideration to the involvement of other immune cell populations, such as natural killer lymphocyte, CD4+ T cells, etc. If Tn may change the rates of lymphocyte recruitment, it could be indirectly associated with immunosuppression. We are investigating further experiments to clarify these subjects.

### Table 2. Univariate and multivariate analyses

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tbody>
<tr>
<td></td>
<td>Risk ratio (95% confidence interval)</td>
<td>Risk ratio (95% confidence interval)</td>
</tr>
<tr>
<td>A. Evaluation of the independence of Tn and CD8 positivity in determining prognosis was done using univariate and multivariate Cox regression analyses. Tn and CD8 were determined to be mutually independent prognostic factors.</td>
<td></td>
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<tr>
<td>Tn+/-</td>
<td>1.947 (1.072-3.5360)</td>
<td>0.0287</td>
</tr>
<tr>
<td>CD8+/-</td>
<td>2.019 (1.096-3.719)</td>
<td>0.0241</td>
</tr>
<tr>
<td>B. Evaluation of possible prognostic factors, including Tn/CD8 status, in 146 patients with colorectal cancer using univariate and multivariate Cox regression analyses. Dukes’ staging and Tn/CD8 status were determined to be independent prognostic factors.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tn+/CD8 [Tn+/CD8+/others]</td>
<td>3.319 (1.833-6.011)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Age (≥63/63)</td>
<td>1.300 (0.706-2.394)</td>
<td>0.3997</td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>1.116 (0.606-2.055)</td>
<td>0.7257</td>
</tr>
<tr>
<td>Histopathologic type (moderate, poor, mucinous/well)</td>
<td>2.286 (1.180-4.428)</td>
<td>0.0142</td>
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<td>Lymphatic invasion (+/-)</td>
<td>2.343 (1.229-4.467)</td>
<td>0.0097</td>
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<tr>
<td>Venous invasion (+/-)</td>
<td>4.071 (2.014-8.231)</td>
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<td>Dukes’ staging</td>
<td>2.874 (2.094-3.643)</td>
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**References**

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