Correlation between Tumor-associated Glycoprotein 72 Mucin Levels in Tumor and Serum of Colorectal Patients as Measured by the Quantitative CA 72-4 Immunoassay

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ABSTRACT

Colorectal tissue biopsies were obtained from 110 patients diagnosed with primary colorectal carcinoma (tumor and normal colonic mucosa samples), 20 patients diagnosed with benign colorectal disease, and 31 healthy donors. The level of expression of tumor-associated glycoprotein 72 (TAG-72) was quantitatively measured in each sample using a double-determinant RIA with monoclonal antibodies B72.3 and CC49 and detecting the sialyl-Tn epitope; this assay was termed CA 72-4. Statistical analysis revealed a significant (approximately 10-fold) increase of TAG-72 expression in the colon tumor biopsies when compared with the expression in normal colonic mucosa from the same patients. A regression analysis revealed a significant correlation (r = 0.459; P < 0.001) between TAG-72 levels measured in biopsies from the tumor lesions and those found in the corresponding normal colonic mucosa. Furthermore, regression analysis showed a significant positive correlation between TAG-72 levels in the tumors and sera of the same patients (r = 0.491; P < 0.001). TAG-72 levels in normal colonic mucosa from healthy donors and patients diagnosed with colorectal cancer were compared. TAG-72 expression was 5-fold higher in the normal mucosa from the colorectal carcinoma patients. No relationship between TAG-72 tumor tissue content and stage of disease was found. Moreover, the correlation between TAG-72 distribution and degree of tumor differentiation observed (P < 0.05) was not any more evident when mucinous carcinomas were excluded. Finally, the results provide further evidence that TAG-72 may be considered an important early marker for colorectal cancer and/or other dysplastic colonic diseases. The statistical correlation between TAG-72 levels in tumors and circulating TAG-72 indicates that patients with elevated levels of serum TAG-72, as measured by the CA 72-4 assay, would be most suited for diagnostic and/or therapeutic intervention with the anti-TAG-72 monoclonal antibodies B72.3 or CC49 or vaccine trials using the sialyl-Tn epitope.

INTRODUCTION

TAG-72 is a carcinoma-associated mucin that was initially identified by MAb B72.3 (1). It has been shown to be overexpressed on a range of carcinomas, including colon, gastric, pancreas, breast, ovary, endometrium, and prostate, as well as non-small cell lung carcinoma (2–6). Overexpression of TAG-72 in nonmalignant tissue has been shown to be restricted to transitional colonic mucosa (histologically normal mucosa adjacent to carcinoma; Ref. 7) and secretory phase endometrium (8). Sialyl-Tn has been shown to be involved in the binding of MAb B72.3 (9). A range of epitopes on the TAG-72 molecule has been detected with the use of "second-generation" MAbs prepared by using purified TAG-72 as an immunogen (10). One of these MAbs, CC49, has been shown to react to a distinct epitope of TAG-72 when compared with B72.3 (10).

MAbs B72.3 and CC49 as well as TAG-72 are currently being used or evaluated in various aspects of the management of a range of human carcinomas: (a) 111In-labeled B72.3 (OncoScint) is approved by the Food and Drug Administration for use in oncological imaging for colorectal cancer and ovarian cancer in conjunction with computed tomographic scanning (11, 12); (b) 125I-labeled CC49 IgG is being used with a hand-held γ-detecting probe for the intraoperative detection of carcinoma lesions (a multicenter Phase III trial has now been concluded; Refs. 13 and 14); (c) 131I-labeled CC49 has been shown to detect more than 90% of carcinoma lesions via γ scanning (15); (d) MAb CC49 labeled with 131I, 90Y, and 177Lu (16–19) are currently in Phase I trials in ovarian, prostate, and breast cancer immunotherapy; (e) the CA 72-4 RIA (composed of MAb B72.3 and CC49) has been shown to detect circulating TAG-72 in colorectal, gastric, and ovarian carcinoma patients (20–23), and the use of CA 72-4 in combination with CEA has been shown to enhance colorectal carcinoma detection (24, 25); (f) synthetic sialyl-Tn is currently being used as a vaccine in Phase II trials in breast cancer patients (26); and (g) the expression of sialyl-Tn either on tumors or in blood has been shown in four independent studies to be an indicator of poor prognosis for ovarian, gastric, and colon cancer (27–31).

Thus, because both MAb B72.3 and CC49 as well as the reactive TAG-72 antigen are currently being analyzed for use in various aspects of cancer management, this study was designed to determine whether a correlation existed between TAG-72 content in tumors and circulating TAG-72 serum levels. An analysis of this issue would facilitate patient selection in various modalities using MAb B72.3 and CC49. A previous study analyzing CEA levels in tumor versus circulating CEA showed absolutely no statistical correlation, i.e., quantitative analysis using RIAs of tumor and serum levels of CEA showed that patients' circulating levels of CEA did not predict CEA content in their tumors.

MATERIALS AND METHODS

Patient Information and Sample Collection. One hundred ten patients with histologically diagnosed primary colorectal adenocarcinoma [65 males and 45 females; mean age, 60.2 ± 1.3 (SE) years; range, 35–92 years] were evaluated. All patients with malignant disease underwent surgery for their...
primary tumors at the Department of Surgery, Regina Elena Cancer Institute, or the Department of Surgery, University of Rome Tor Vergata. Malignant colorectal disease was pathologically staged according to Dukes’ classification: stage A, n = 12; stage B, n = 42; stage C, n = 36; and stage D, n = 20.

Presurgical serum samples and multiple biopsies (minimum of five) of histologically confirmed neoplastic tissues and normal mucosa were obtained at the time of surgery, immediately frozen in liquid nitrogen, and subsequently evaluated for TAG-72 content. All carcinoma specimens were free of necrotic areas and contained at least 50% malignant cells by histopathology. All specimens designated as normal mucosa from carcinoma patients contained at least 80% histologically normal colonic mucosa. Thirty-one healthy donors and 20 patients with benign colorectal disease entered into the study. The healthy donors included subjects enrolled in an institutional program of surveillance for gastrointestinal cancer. TAG-72 content in healthy donors was used as a control. The quantitative evaluation of TAG-72 tissue content was performed on whole protein extracts obtained from all the biopsies.

Preparation of Tissue Protein Extracts. The procedure for the protein extraction has been reported previously (10). Briefly, all colorectal tissues were homogenized with an Omni 1000 homogenizer (Omni International, Waterbury, CT) at high speed (20,000 rpm). The tissues were initially resuspended at a ratio of 1:10 g tissue/ml in buffer containing 10 mM Tris-HCl (pH 7.2) and 0.2 mM CaCl₂ (extraction buffer). The homogenate was centrifuged at 1,000 × g for 10 min, and the supernatant was removed and sonicated at 4°C for 1 min at 15-s intervals. The sonicate was centrifuged at 10,000 × g for 10 min, and the protein concentration of the supernatant was determined (32).

Determination of TAG-72 Content in Tissue Samples. TAG-72 content was measured in protein extracts from colorectal tissue biopsies of normal donors and patients diagnosed with benign or malignant colorectal disease. In the case of cancer patients, tissue samples from the tumor lesion and from normal mucosa (approximately 10 cm from the tumor) were evaluated. All protein extracts obtained from these tissues were initially diluted with extraction buffer to a protein concentration of 1.0 mg/ml. TAG-72 was measured in a 100-μl sample of the protein extracts in duplicate, using the CA 72-4 DDRIA kit (kindly provided by Centocor, Inc., Malvern, PA; Ref. 20). All samples exceeding the standard curve were serially diluted in protein extraction buffer, and the TAG-72 evaluation was repeated. In all assays, extracts from control carcinoma samples were included, as well as control low and high TAG-72 standards. The value for each sample was calculated as the mean of at least two determinations of three different biopsies from the same sample. The limit of normality for TAG-72 tissue content was 5 units/mg protein and was calculated as 2 SD above the mean TAG-72 content of colorectal biopsies obtained from normal donors.

Determination of TAG-72 Content in Serum Samples. Serum samples from cancer patients were drawn within 1 week before surgery; serum samples from patients with benign disease were drawn just prior to endoscopy. Serum samples were coded and stored at −40°C until assayed. Serum TAG-72 levels were determined using the CA 72-4 DDRIA kit, and a cutoff value of 6 units/mI was used (20).

STATISTICAL ANALYSIS. Statistical analysis was performed using the Mann-Whitney U test, the Kruskal-Wallis test, and the χ² test for group comparison. Only results with P < 0.05 were regarded as significant. All calculations were made using the STATVIEW software package.

RESULTS

Tumor and normal colorectal tissue biopsies were obtained from 110 patients diagnosed with primary colorectal carcinoma; tissue biopsies were also collected from 20 patients diagnosed with benign colorectal disease and from 31 healthy donors. All tissue samples were processed as described in “Materials and Methods,” and TAG-72 levels were determined using the quantitative CA 72-4 DDRIA assay. Table 1 summarizes the ranges and means of TAG-72 content measured in these tissue extracts. Tissue biopsies from healthy individuals contained approximately 3.9 TAG-72 units/mg protein, with a range of 1.4–8.0 units/mg protein (Table 1). Patients entered into the study and diagnosed with benign colorectal lesions were found to express TAG-72 levels approximately 6-fold higher than those measured in biopsies from healthy donors. As expected, TAG-72 levels were highest in tissue extracts prepared from the histologically confirmed carcinoma biopsies. The mean TAG-72 levels in these samples were >50-fold higher (i.e., 208.0 ± 96.6 units/mg protein) than those of the healthy donors. Interestingly, the mean TAG-72 levels in the normal mucosa biopsies from the same patients diagnosed with colorectal carcinoma were 21.0 ± 8.3 units/mg protein, which were similar to the amounts of TAG-72 measured in the biopsy samples from the benign colorectal lesions.

Fig. 1 illustrates an analysis of TAG-72 levels in the four groups of tissue samples (normal mucosa from healthy donors, benign lesions, and both normal mucosa and tumor samples from colorectal cancer). A cutoff value of 5 units/mg protein to discriminate between TAG-72-positive and TAG-72-negative tissue samples was established. The 5-units/mg protein cutoff value was calculated as the mean (3.9 units/mg protein) + 2 SD from the mean (SD, 0.5 units/mg protein) of the TAG-72 content measured in the colorectal tissue samples obtained from healthy donors. Interestingly, the tissue TAG-72 cutoff value is virtually identical to that for serum TAG-72 (i.e., 6 units/ml; Ref. 20). Two of 31 (6.5%) colorectal tissue samples from healthy donors contained TAG-72 levels in excess of 5 units/mg protein (Fig. 1). The percentage of carcinoma tissue biopsies collected from the 110 carcinoma specimens in which TAG-72 levels were >5 units/mg protein was 86.4% (95 of 110 samples), whereas that for the histologically normal tissue biopsies from the same patients was 40.9% (45 of 110 samples). Fourteen of 20 tissue samples (70%) taken from patients with benign colorectal disease had TAG-72 levels that exceeded the 5-units/mg protein cutoff (Fig. 1). A correlation between the antigen content and the degree of dysplasia was observed. In fact, the higher antigen expression was observed in the six cases with severe dysplasia, whereas all six hyperplastic polyps showed levels of the antigen in the normal range (Fig. 1).

TAG-72 levels were compared from the carcinoma and normal colonic mucosa (5–10 cm from the tumor) tissue biopsies from the same patient. As shown in Fig. 2, a regression analysis revealed a significant correlation (r = 0.458; P < 0.001) between TAG-72 levels measured in biopsies taken from the tumor center and those found in the normal colonic mucosa obtained 5–10 cm from the tumor margin.

Previous clinical trials indicated a correlation between positive tumor localization of radioimmunoconjugated anti-TAG-72 MAb (i.e., B72.3 and CC49) in patients diagnosed with colorectal carcinomas who also had positive serum TAG-72 levels (11). TAG-72 levels

<table>
<thead>
<tr>
<th>Patients</th>
<th>No. of samples</th>
<th>Histological diagnosis</th>
<th>TAG-72 content (units/mg protein)</th>
<th>Range</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>31</td>
<td>Normal¹</td>
<td>1.4–8.0</td>
<td>3.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Benign colorectal disease</td>
<td>20</td>
<td>Benign lesion²</td>
<td>2.4–72.6</td>
<td>23.9 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td>110</td>
<td>Carcinoma³</td>
<td>1.3–8555.0</td>
<td>208.0 ± 96.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>Normal⁴</td>
<td>1.3–350.0</td>
<td>21.0 ± 8.3</td>
<td></td>
</tr>
</tbody>
</table>

¹ At least three different colorectal biopsies were taken, and TAG-72 was measured using the CA 72-4 RIA. See “Materials and Methods” for the explanation of benign patients and healthy donors.

² All carcinoma samples were biopsied in the central area of the lesion in an attempt to avoid necrosis. From those same patients, biopsies from the normal mucosa were taken 7.5–10 cm distal from the tumor.

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measured in carcinoma biopsies were compared with the corresponding TAG-72 serum levels from the 110 patients diagnosed with colorectal carcinoma. A linear regression analysis (Fig. 3) revealed a significant correlation ($P < 0.001$) between TAG-72 tumor and serum levels.

TAG-72 expression in carcinoma lesions did not correlate with Dukes' stage. Higher tissue TAG-72 levels were observed in well-differentiated tumors when evaluated on the basis of degree of differentiation (well versus moderate versus poor: $P < 0.05$; Fig. 4). When the mucinous carcinoma cases were removed, however, so was the statistical difference between TAG-72 tissue levels and degree of differentiation ($P$, not significant).

Patients diagnosed with primary colorectal carcinoma were subsequently divided into two groups according to TAG-72 serum levels: patients with negative (i.e., $<6$ units/ml) and positive (i.e., $>6$ units/ml) serum TAG-72 levels. Using the 5-units/mg protein cutoff value for tissue TAG-72 levels, a significant difference of TAG-72 content was found between the two groups ($P < 0.005$). Clearly, those individuals who had the highest serum TAG-72 levels also had the highest TAG-72 tumor content. This finding agrees with the positive correlation between tissue and serum TAG-72 content reported in Fig. 3. The frequency distribution of TAG-72 in these two different subgroups was evaluated. The percentage of cases with high TAG-72 tumor tissue content and positive serum levels was significantly different from that observed in patients with negative serum TAG-72. In fact, approximately 60% of the patients with positive TAG-72 serum levels had a tissue content greater than 100 units/mg protein, compared with the 7% of the patients with negative serum levels.

**DISCUSSION**

The present study quantitatively measured TAG-72 levels in a variety of colonic mucosa tissue samples using the CA 72-4 DDRIA. The results clearly show a differential level of TAG-72 expression particularly between the carcinoma lesions and normal colonic mucosa samples taken from a group of 110 patients diagnosed with primary adenocarcinoma. The concentration of TAG-72 (units/mg protein) was approximately 10-fold higher in the carcinoma samples than the normal colonic mucosa samples that were biopsied 10 cm from the tumor. Previous immunohistochemical studies using B72.3

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**Fig. 1.** TAG-72 content in colorectal tissues from healthy donors ($n = 31$), patients diagnosed with benign diseases ($n = 20$) [hyperplastic polyps (△), tubulovillous adenomas with low or moderate dysplasia (■), and tubulovillous adenomas with severe dysplasia (□)], and patients diagnosed with colorectal cancer ($n = 110$).

**Fig. 2.** Linear regression analysis of TAG-72 content in tumor lesions and corresponding histologically confirmed normal mucosa from colorectal adenocarcinoma patients. ○, TAG-72 levels in tumor and normal colonic mucosa from the same patient.
QUANTITATIVE TAG-72 IN NORMAL AND MALIGNANT COLONIC MUCOSA

and CC49 staining of TAG-72 have reported elevated TAG-72 expression in primary and metastatic colorectal tumors as well as the transitional colonic mucosa compared with the expression level in normal colonic mucosa (2–7, 33). Although immunohistochemical analysis of TAG-72 expression provides important information about antigen expression with respect to the percentage of cells expressing TAG-72 and tissue compartments and architecture, its quantitative aspects are limited. Moreover, in many cases, biopsies of metastatic lesions are not available for biopsy and immunohistochemical analyses. Thus, in those cases, serum TAG-72 levels could serve as a surrogate marker for TAG-72 expression in the metastatic lesion.

The ability to quantitate actual TAG-72 levels within the different colonic mucosa tissue extracts provides another perspective on the biology of that human tumor antigen. For example, it was intriguing to find that TAG-72 levels in the normal colonic mucosa isolated from the patients diagnosed with primary colon cancer were approximately 5-fold higher than those measured from normal colonic mucosa taken from healthy donors. In fact, using the tissue cutoff of 5 units TAG-72/mg protein, of the 110 normal colonic mucosa samples from the carcinoma patients, 45 had elevated TAG-72 levels, compared with 2 of 31 normal colonic tissue samples from the healthy donors. Immunohistochemical evidence has shown an elevation of TAG-72 expression within the transitional colonic mucosa, which, in that study, was defined as normal-appearing mucosa adjacent to the tumor (7). The

Fig. 3. Linear regression analysis of serum TAG-72 levels and TAG-72 tumor tissue content in colorectal adenocarcinoma patients. •, TAG-72 levels in serum and tumor tissue from the same patient.

Fig. 4. Relationship between TAG-72 tumor tissue content and degree of tumor differentiation. Circles, individual patients; ○, patients diagnosed with mucinous colorectal adenocarcinoma.
present findings indicate that elevated TAG-72 levels can be demonstrated within a region 10 cm from the tumor site. Moreover, TAG-72 levels from colonic mucosa that was diagnosed as nonmalignant but containing benign disease were also elevated when compared with normal colonic mucosa from healthy donors. These results are in agreement with a previous study (34) demonstrating that 75% of colorectal premalignant lesions with severe dysplasia overexpressed TAG-72, whereas none of the nonneoplastic lesions was positive. Therefore, the authors concluded that elevated expression of TAG-72 in the colonic epithelium may be considered an early marker of malignant transformation (34). In the case of patients diagnosed with colorectal carcinoma, an elevation of TAG-72 10 cm from the tumor site suggests that: (a) the so-called “transformation zone,” which is identified by elevated TAG-72 levels, may extend farther from the tumor site than initially thought; and (b) if TAG-72 is, indeed, an early marker of malignant transformation, its elevation for 10 cm away from the tumor site provides an additional argument to consider colorectal cancer an organ disease, not just localized at individual sites along the colon. One can only speculate why TAG-72 levels were increased in the histologically normal colonic mucosa samples taken from patients diagnosed with colorectal cancer. Several possibilities include early genetic and/or epigenetic changes (i.e., paracrine effects) within the colonic mucosa cells. Those changes may alter the normal glycosyltransferase activity within the colonic mucosa cells and result in inappropriate carbohydrate substitution of the mucin polypeptide core, which leads to TAG-72 expression, as defined by the appearance of the B72.3-reactive sTn antigen site.

Quantitative analysis of TAG-72 levels in the aforementioned tissues also provides some insight into a potential “therapeutic index” between actual TAG-72 levels in tumor and normal colonic tissue and ongoing interest to exploit TAG-72 as a target for passive and active immunotherapy. It should be noted that the approximate 10-fold difference between TAG-72 levels in primary colorectal tumor and normal tissues from the patients diagnosed with colon carcinoma is probably an underestimation, because the tumor tissues included some nonmalignant areas. In support of this, TAG-72 has been shown in numerous studies to be an excellent target for radioimmunon conjugates, including radiolabeled B72.3 and CC49 (11, 12, 16—19, 35). MAb B72.3, known as In-CYT-103, has received Food and Drug Administration approval for routine assessment, in conjunction with computed tomographic scanning, of disease spread in patients suspected of colorectal or ovarian cancer (11, 12). Iodine-labeled CC49 has been successfully used to identify occult tumors intraoperatively with a hand-held y-detecting probe (13, 14). An important finding shared by all of those early clinical trials is the lack of uptake of anti-TAG-72 MAbs by normal tissues. Therefore, the ability of anti-TAG-72 MAbs to target colorectal and other tumors in those numerous clinical trials underscores the importance of the differential TAG-72 expression levels between tumor and nontumor tissues. Some of those same clinical trials, which were designed to evaluate the tumor targeting of the anti-TAG-72 MAbs, also evaluated whether the presence of serum TAG-72 was any indicator of the ability of B72.3 or CC49 to target tumors. Arguments suggested that serum tumor antigen levels would act as a reservoir and decrease the bioavailability of the MAb to the tumor. Retrospective analysis of the tumor-targeting ability of B72.3 or CC49 in patients with or without serum TAG-72 levels clearly showed that low to moderate serum TAG-72 levels were actually predictive of positive MAB tumor targeting (15, 18). Those empirical results can now be explained by the findings of the present study of a high correlation between serum and tumor TAG-72 levels. Therefore, serum TAG-72 levels may be used to select patients for immuno scintigraphy or, perhaps, MAB-based immunotherapy.

The studies reported here contrast with the results observed with tumor versus serum levels of CEA. In those studies, absolutely no statistical correlation was observed between serum CEA levels and quantitative analysis of CEA in tumors. To our knowledge, no other tumor-associated antigens, such as c-erbB2, muc-1, and CA 125, have been evaluated for correlations between quantitative levels in tumor versus serum. The results presented here demonstrating the statistical correlation between tumor and serum levels of TAG-72 suggest that optimal results using anti-TAG-72 MAbs such as CC49 or B72.3 (OncoScint) for use in oncological imaging, radioimmunoguided surgery, or immunotherapy would be obtained in those patients with higher serum levels of TAG-72 as analyzed using the CA 72-4 DDRIA. Finally, elevated sialyl-Tn serum levels have been shown to be poor prognostic indicators in ovarian and gastric cancer (27—29). Moreover, elevated levels of sialyl-Tn in colorectal and gastric tumors, as measured by immunohistochemistry, have been shown to be poor prognostic indicators (30, 31). The results reported here suggest that either retrospective or prospective studies should be carried out to determine whether a correlation exists between elevated levels of circulating sialyl-Tn, as measured by the CA 72-4 assay, and poor prognosis in patients with colorectal carcinoma.

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REFERENCES


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