A More Specific, Simpler Radioimmunoassay for Carcinoembryonic Antigen, with Use of Monoclonal Antibodies

A solid-phase, two-site monoclonal antibody radioimmunoassay for carcinoembryonic antigen in serum or plasma has been developed. Two monoclonal antibodies are used: 3d6, which is immobilized on polystyrene beads, reacts with high-molecular-mass CEA; the other, C4, with apparently restricted reactivity toward gastrointestinal tract and mammary carcinomas, is labeled with 125I. The assay consists of incubating 0.2 mL of serum both with 3d6-coated beads and 125I-labeled C4 at room temperature for 18 h. The CEA standard is calibrated against WHO international standard (73/601). Advantages of this assay include: (a) no heat or acid pre-treatment of samples; (b) linear response over a wider functional range, 0 to 150 µg/L, requiring fewer calibration points; and (c) no interference by glycosaminoglycans. Average inter- and intra-assay reproducibilities (CVs) are <10%; analytical recovery of CEA was 94 to 107%. CEA of <0.5 µg/L can be detected. The mean concentration of CEA in serum from healthy individuals is 0.97 (SD 1.18) µg/L; only 3% of the sera tested had concentrations >3.0 µg/L. On comparing this assay with a polyclonal RIA, we found similar assay sensitivity for colorectal carcinoma but fewer false-positive results for sera from patients with benign liver and bowel diseases.

Additional Keyphrases: reference interval · colorectal carcinoma · cancer · cutoff value · sex- and age-related values

Since first described by Gold and Freedman (1), the role of carcinoembryonic antigen (CEA) as a tumor-associated marker in managing cancer of the colon (e.g.) has been widely investigated (2, 3). Sequential measurement of plasma CEA pre- and post-operatively has been evaluated as a tool to monitor recurrence or progression of disease, response to the course of adjuvant therapy, and thoroughness of resection of malignancy.

Existing commercial polyclonal CEA assays have been used extensively in aiding cancer management, but their lack of cancer specificity and the need for cumbersome pre-treatment of specimens to separate CEA from nonspecific plasma proteins have limited their clinical usefulness. A major goal in current research, therefore, is to design an immunoassay for CEA that is simpler and more cancer specific.

Hybridoma technology (4) has been used by several research groups (5–11) to raise monoclonal antibodies to CEA. Because they are highly specific, monoclonal antibodies have the potential to differentiate among immunologically related antigenic determinants—e.g., CEA and cross-reactive antigens to CEA in the plasma such as nonspecific cross-reacting antigen, normal fetal antigen-2, biliary glyco-

protein-1, and tumor-extracted cross-reacting antigen (12–15). Therefore, a simplified and more specific immunoassay for CEA might be developed by using monoclonal antibodies.

Here we report the development of a two-site monoclonal antibody radioimmunoassay (MAb CEA-RIA) for quantifying CEA in serum or plasma. This assay is simple, sensitive, and reproducible; it is not subject to interference by glycosaminoglycans; and it has a low rate of false-positive results for normal subjects or those with benign diseases, especially ulcerative colitis or cirrhosis of the liver.

Materials and Methods

Monoclonal anti-CEA antibodies. Two monoclonal antibodies were used in assay development. One of them, 1116N6-3d6-35 (designated here as 3d6), was derived from the fusion of P3 × 63 Ag8 mouse myeloma cells with splenocytes of mice immunized with SW 1116 colorectal carcinoma cells. Antibody 3d6 is of the IgG1 subclass. The other monoclonal antibody, C420-32 (here designated C4), was derived from the fusion of 663 mouse myeloma cells immunized with splenocytes of mice immunized with SW 1223 colorectal carcinoma cells. C4 is of the IgG2a subclass.

The generation, characterization, and reactivity of these two monoclonal antibodies have been previously described (6, 11). 3d6 reportedly binds to purified CEA, and it precipitates a polypeptide of 180 000 Da. C4 has a restricted reactivity towards gastrointestinal tract and mammary carcinomas.

Both murine hybridomas were obtained from The Wistar Institute, Philadelphia, PA. Hybridoma cells were cloned, grown in tissue culture, and used to prepare ascitic fluid in BALB/c mice primed with pristane (Aldrich Chemical Co., Milwaukee, WI 53201) (16). Immunoglobulins were purified from ascites by affinity chromatography on protein A-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ 08854) (17). The isolated immunoglobulins were pure, as judged from sodium dodecyl sulfate/polyacrylamide gel electrophoresis, isoelectric focusing, and immunoelectrophoresis (data not shown).

Preparation of immunoreagents. Purified C4 was radiiodinated with the 125I-labeled N-hydroxysuccinimide ester of p-hydroxyiodophenyl propionic acid (Amersham Corp., Arlington Heights, IL 60005) (18), according to the manufacturer's recommended procedure. Specific activity of the 125I-labeled C4 ranged from 10 to 13 Ci/g. Polystyrene beads, 6.35 mm in diameter (Precision Plastic Ball Co., Chicago, IL 60641), were coated with 3d6 and dried (19). CEA assay standards were prepared from supernates of a cultured human colorectal adenocarcinoma-derived cell line SW 1116 (20) diluted into pooled human CEA-negative serum.

The assay standards were calibrated against the World Health Organization (WHO) Standard for CEA obtained from the National Institute for Biological Standards and Control, Holly Hill, London, NW3 6RB, U.K. (coded 73/601, and containing 10 µg of CEA (21)]. 1.04 µg of the CEA assay

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standard per liter being equivalent to 1.0 μg of the WHO standard per liter.

**Assay procedures.** Experimental samples were assayed in duplicate with a "simultaneous sandwich" radioimmunometric assay. We added 0.2 mL of specimen, standards, or positive control to the reaction tray along with one 3d6-coated bead and 900 000 dpm (0.1 mL) of 125I-labeled C4 in 100 mmol/L Tris buffer, pH 7.5, containing 250 mL of calf serum (Sterile Systems, Inc., Logan, UT 84321) and 10 mL of normal mouse serum (Hazelton Dutchtland, Inc., Denver, PA 17517) per liter.

After incubation at room temperature (18–22 °C) for 18 h, the beads were washed three times with water and the bound radioactivity was counted in a gamma counter. An assay standard curve was constructed by plotting CEA concentration in micrograms per liter vs counts per minute. Experimental results, micrograms of CEA per liter, were derived by comparison with the standard curve.

**Commercial CEA controls.** CEA check levels 1, 2, 3, and 4, and CEA control plasma levels 1, 2, and 3 were purchased from CEA Lab Products, Denver, CO 80216; and from Pacific HemoNostasis, Bakersfield, CA 93309, respectively.

**Glycosaminoglycans.** Hyaluronic acid, potassium salt, from the human umbilical cord and dextran sulfate, sodium salt, were purchased from the U.S. Biochemical Corp., Cleveland, OH 44128. Heparin, Grade II, sodium salt, from porcine intestinal mucosa, and chondroitin sulfate, Grade III, sodium salt, from whale cartilage, were from Sigma Chemical Co., St. Louis, MO 63178.

**Patients’ samples.** Patients’ samples were from the National Cancer Institute, the Mayo Clinic Serum Bank, and the Memorial Sloan-Kettering Institute.

Serum samples from 679 healthy blood donors were obtained from Dr. J. Menitove (Southeastern Blood Center, Milwaukee, WI). All of these donors qualified as blood donors and were negative when tested for hepatitis B surface antigen. Data concerning age and sex, provided by Dr. Menitove, are shown below in Figure 4.

**Comparison immunoassay.** Sera tested in the MAb CEA-RIA were also assayed in parallel with a conventional polyclonal CEA-RIA (Abbott Laboratories, North Chicago, IL). In both assays, a CEA concentration ≥3 μg/L was used as the cutoff value, that value used to discriminate between positive and negative samples.

**Results**

**Assay conditions.** We investigated the feasibility of using antibodies 3d6 and C4 as the solid-phase capturing antibody and the radioisotope-labeled tracer antibody, respectively. A sensitive assay was obtained when we used 3d6 on the solid phase and C4 as the tracer antibody. The strongest dose–response relationship was obtained when 3d6-coated bead, specimen, and C4 tracer were assayed in the "simultaneous sandwich" format (i.e., with one incubation step) at room temperature for 18 h. Assays with the "forward sandwich" format (i.e., two incubation steps) at room temperature or higher temperatures (37 or 45 °C) for 2 h to as long as 24 h yielded a much weaker dose–response curve as compared with the simultaneous assay format. No acid or heat treatment of the specimen before assay was required. Serum or plasma samples could be used directly, i.e., without any pretreatment, in the measurement of CEA. Figure 1 shows a typical dose–response standard curve.

**Sensitivity and linearity.** The standard curve is linear from zero to approximately 150 μg of CEA per liter, and only at concentrations of CEA >2000 μg/L is a decrease in assay signal observed (Figure 2). The measured CEA values are independent of dilution. The minimum detectable concentra-

![Fig. 1. Typical standard curve for the present assay](image1)

![Fig. 2. Dose–response curve and "hook-effect" in the present assay](image2)

<table>
<thead>
<tr>
<th>Table 1. Analytical Recovery in the Present Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CEA added</strong></td>
</tr>
<tr>
<td>μg/L</td>
</tr>
<tr>
<td>6.9</td>
</tr>
<tr>
<td>13.8</td>
</tr>
<tr>
<td>27.5</td>
</tr>
<tr>
<td>55.0</td>
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<tr>
<td>110.0</td>
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Table 2. Intra- and Interassay Reproducibility of the Present Assay

<table>
<thead>
<tr>
<th></th>
<th>Intra-assay</th>
<th>Interassay</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>µg/L</td>
<td>µg/L</td>
<td>%</td>
</tr>
<tr>
<td>2.0</td>
<td>0.2</td>
<td>10.0</td>
</tr>
<tr>
<td>3.2</td>
<td>0.2</td>
<td>5.4</td>
</tr>
<tr>
<td>3.7</td>
<td>0.3</td>
<td>5.4</td>
</tr>
<tr>
<td>5.9</td>
<td>0.3</td>
<td>5.1</td>
</tr>
<tr>
<td>11.8</td>
<td>0.5</td>
<td>4.2</td>
</tr>
<tr>
<td>24.9</td>
<td>0.8</td>
<td>3.2</td>
</tr>
<tr>
<td>53.4</td>
<td>2.9</td>
<td>5.4</td>
</tr>
<tr>
<td>120</td>
<td>7.1</td>
<td>5.9</td>
</tr>
</tbody>
</table>

* Eight different sera; n = 10 replicates throughout.

giving false-positive results for CEA (25). To examine their effect on assay results, we added high concentrations of glycosaminoglycans to human CEA-negative serum and to serum containing 11.0 µg of CEA per liter. The assay results are illustrated in Table 3; we saw no false CEA measurements, nor any significantly decreased CEA measurements.

Correlation with a conventional polyclonal radioimmunooassay. To examine the correlation between the MAB CEA-RIA (y) and Abbott CEA-RIA (x), we analyzed a series of CEA-containing specimens by both assays. The specimens included (a) a serial dilution of high-CEA serum from a colorectal cancer patient and (b) seven commercial CEA standard samples. The correlation coefficient was 0.983 and the linear regression equation was y = 1.01x − 0.629. The correlation between these two assays was good (Table 4).

Reference intervals. Figure 3 illustrates the frequency distribution of CEA values for 679 normal blood donors. The measured CEA ranged from 0 to 4.6 µg of CEA per liter (mean 0.97, SD 1.18 µg/L). Only 3% of the population tested had a CEA value exceeding 3 µg/L. As indicated in Figure 4, the mean concentration of CEA did not vary significantly with the age of the donor (p < 0.05, Student's t-test). In this group of donors, the mean concentration of CEA in the sera of male donors was significantly higher than for female donors in all age groups (p > 0.05) except in those 40–49 years old.

Determination of serum CEA values in patients. Sera from 480 patients with benign diseases or advanced cancers were studied. Table 5 summarizes the comparison of the MAB CEA-RIA with Abbott's CEA-RIA. The MAB CEA-RIA gives fewer false-positive results for samples from benign-disease patients, especially those with nonmalignant liver and gastrointestinal diseases.

Table 3. Effect of Glycosaminoglycans on Assay of CEA in Human Serum with and without Detectable CEA

<table>
<thead>
<tr>
<th>GAG concentration, mg/L</th>
<th>CEA, µg/L, measured in human serum containing 11.0 µg of CEA per liter*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin sulfate</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>10.4</td>
</tr>
<tr>
<td>40</td>
<td>10.9</td>
</tr>
<tr>
<td>60</td>
<td>10.8</td>
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<tr>
<td>80</td>
<td>9.9</td>
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<tr>
<td>100</td>
<td>10.0</td>
</tr>
<tr>
<td>300</td>
<td>10.9</td>
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<tr>
<td>Dextran sulfate</td>
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</tr>
<tr>
<td>3.1</td>
<td>11.4</td>
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<tr>
<td>6.3</td>
<td>10.8</td>
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<tr>
<td>12.5</td>
<td>10.0</td>
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<tr>
<td>25</td>
<td>10.3</td>
</tr>
<tr>
<td>50</td>
<td>11.0</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>11.0</td>
</tr>
<tr>
<td>20</td>
<td>10.8</td>
</tr>
<tr>
<td>39</td>
<td>11.4</td>
</tr>
<tr>
<td>78</td>
<td>10.5</td>
</tr>
<tr>
<td>313</td>
<td>10.4</td>
</tr>
<tr>
<td>625</td>
<td>10.0</td>
</tr>
<tr>
<td>1250</td>
<td>9.7</td>
</tr>
<tr>
<td>Heparin</td>
<td></td>
</tr>
<tr>
<td>9.5</td>
<td>11.0</td>
</tr>
<tr>
<td>19</td>
<td>11.7</td>
</tr>
<tr>
<td>38</td>
<td>12.0</td>
</tr>
<tr>
<td>76</td>
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<tr>
<td>150</td>
<td>10.9</td>
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<td>300</td>
<td>10.6</td>
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<td>600</td>
<td>9.9</td>
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<tr>
<td>1500</td>
<td>10.1</td>
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<tr>
<td>3000</td>
<td>9.9</td>
</tr>
<tr>
<td>6000</td>
<td>9.4</td>
</tr>
</tbody>
</table>

* n = 4 for each. In a parallel series in which serum negative for CEA was tested (also n = 4), results were consistently zero.

Table 4. Correlation between Results by the Present Assay and the Abbott CEA-RIA for Various CEA-Containing Samples

<table>
<thead>
<tr>
<th>CEA-containing samples from cancer patients</th>
<th>Present RIA</th>
<th>Abbott CEA-RIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/L</td>
<td>µg/L</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>116.0</td>
<td>98.1</td>
</tr>
<tr>
<td>2</td>
<td>63.6</td>
<td>61.8</td>
</tr>
<tr>
<td>3</td>
<td>33.1</td>
<td>36.9</td>
</tr>
<tr>
<td>4</td>
<td>19.2</td>
<td>21.6</td>
</tr>
<tr>
<td>5</td>
<td>10.1</td>
<td>12.3</td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>6.6</td>
</tr>
<tr>
<td>7</td>
<td>3.5</td>
<td>3.6</td>
</tr>
<tr>
<td>8</td>
<td>1.2</td>
<td>1.2</td>
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</table>

<table>
<thead>
<tr>
<th>CEA commercial control</th>
<th>Present RIA</th>
<th>Labeled mean value</th>
<th>Labeled range</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/L</td>
<td>µg/L</td>
<td>range</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.7</td>
<td>2.0</td>
<td>(1.6–2.5)</td>
</tr>
<tr>
<td>2</td>
<td>5.7</td>
<td>5.9</td>
<td>(5.2–6.7)</td>
</tr>
<tr>
<td>3</td>
<td>12.6</td>
<td>13.8</td>
<td>(11.8–15.9)</td>
</tr>
<tr>
<td>4</td>
<td>34.1</td>
<td>34.2</td>
<td>(29.8–38.6)</td>
</tr>
<tr>
<td>5</td>
<td>5.2</td>
<td>5.8</td>
<td>(4.8–6.8)</td>
</tr>
<tr>
<td>6</td>
<td>20.2</td>
<td>19.7</td>
<td>(17.2–22.2)</td>
</tr>
<tr>
<td>7</td>
<td>94.2</td>
<td>110</td>
<td>(93.0–127.0)</td>
</tr>
</tbody>
</table>

\[y = 1.01x\ (Abbott\ CEA-RIA) - 0.629, r = 0.983.\]

Fig. 3. Frequency distribution of CEA concentrations in normal blood donors.
Fig. 4. CEA concentration in 679 normal blood bank donors, by age and sex

Discussion

Of the many "tumor markers" for human cancer that currently are under development and investigation, CEA has had the most widespread clinical application. However, a major weakness of CEA assay is its nonspecificity for malignancy. Increases in serum CEA have been associated with various conditions and benign disease such as age, cigarette smoking, colonic polyposis, inflammatory bowel disease, and hepatobiliary disease (26). This makes it difficult to accurately discriminate between cancer and benign disease. In addition, most of the commercial polyclonal immunodiagnostic tests for CEA require extensive pre-assay specimen treatment to remove non-specific reacting plasma proteins from the assay. Although improved procedures have recently been reported (27), they still involve additional steps too cumbersome for large-scale application.

Here we have reported our efforts to use two monoclonal anti-CEA antibodies with different epitope specificities to develop a simplified and improved immunodiagnostic test for CEA. The assay has several distinct advantages over conventional polyclonal CEA kits. It totally eliminates the need for specimen-extraction procedures, and so obviates the awkward neutralization, dialysis, ultrafiltration, gel chromatography, and (or) centrifugation steps. The assay is easy to perform and involves only one incubation step, at room temperature. It is not subject to interference by glycosaminoglycans. It is sensitive and reproducible. Standard curves are essentially linear over a relatively wide range of CEA concentrations; thus samples containing abnormally high concentrations of CEA must less commonly be diluted before assay. Recoveries of CEA are excellent over a wide assay range. This allows more-accurate estimation of CEA concentrations in highly positive samples by simple dilution analysis.

The antigenic heterogeneity of the CEA has been attributed to at least five or six different molecules (11, 28, 29). No monoclonal antibodies that react with these molecules have been reported to be tumor specific. However, there are differences in cross reactivity toward epitopes shared by normal tissues. Thus, proper selection and combination of different monoclonal antibodies may provide improved assay specificity. The two monoclonal antibodies we used in our assay, 3d6 and C4, though not absolutely specific for malignancy, do have restricted cross reactivity toward normal and benign tissues (11). When MAB 3d6 and C4 were used in combination in a radioimmunoadsorbent from more than 600 healthy blood-bank donors, the mean value was 0.97 μg/L, with less than 3% of the samples having CEA concentrations >3.0 μg/L. Thus the present assay has a lower reference value, 3.0 μg/L, as compared with 5.0 μg/L, the value recommended in most conventional CEA assays. When we assayed sera in parallel with the MAB CEA-RIA and a conventional polyclonal CEA-RIA—sera from over 480 patients with malignant and benign diseases—we observed similar sensitivity towards colorectal adenocarcinoma but fewer false-positive sera from patients with various benign diseases. This was especially obvious with nonmalignant liver disease, pancreatic diseases, and benign gastrointestinal diseases such as ulcerative colitis (MAB CEA-RIA, 2/11, or 18%; CEA-RIA, 8/11, or 73%, data included with gastrointestinal benign disease in Table 5). Additionally, the mean CEA value by the present method for sera from 31 heavy smokers was 0.99 μg/L, with one sample of 3.2 μg/L, nearly identical to the mean value for the healthy nonsmoker population. Consequently, our assay deserves further evaluation, especially among longitudinally collected serum samples from cancer patients, to determine how well antigen concentration correlates with clinical course.

We thank Tony Green and Jeff Wagner for their excellent technical assistance, and Beverly Dixon for her computer and statistical analyses of the data. We thank Dr. J. Menitove for the healthy blood-bank donor sera. Our thanks also to G. Doughten for her excellent assistance with the manuscript.

Table 5. Comparison of Results by the Present Assay and Abbott’s CEA-RIA in Patients with Cancer or Benign Disease

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. tested</th>
<th>No. positive in present RIA (CEA &gt; 3 μg/L)</th>
<th>Percent</th>
<th>No. positive in Abbott’s RIA (CEA &gt; 3 μg/L)</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorectal adenocarcinoma*</td>
<td>79</td>
<td>42</td>
<td>53</td>
<td>46</td>
<td>58</td>
</tr>
<tr>
<td>Advanced</td>
<td>79</td>
<td>42</td>
<td>53</td>
<td>46</td>
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<tr>
<td>Localized</td>
<td>24</td>
<td>7</td>
<td>29</td>
<td>6</td>
<td>25</td>
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<tr>
<td>Pancreatic adenocarcinoma</td>
<td>37</td>
<td>13</td>
<td>35</td>
<td>20</td>
<td>54</td>
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<tr>
<td>Lung adenocarcinoma</td>
<td>20</td>
<td>9</td>
<td>45</td>
<td>9</td>
<td>45</td>
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<tr>
<td>Breast adenocarcinoma</td>
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<td>11</td>
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<tr>
<td>Stomach adenocarcinoma</td>
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<td>29</td>
<td>2</td>
<td>29</td>
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<tr>
<td>Benign diseases</td>
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<td></td>
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<tr>
<td>Liver (cirrhosis and hepatitis)</td>
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<td>6</td>
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<td>28</td>
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<td>Gastrointestinal*</td>
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<td>10</td>
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<td>Benign nongastrointestinal diseases</td>
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<td>2</td>
<td>3</td>
<td>7</td>
<td>10</td>
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<td>Other diseases</td>
<td>70</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>13</td>
</tr>
</tbody>
</table>

*Advanced colorectal cancer corresponds to Dukes' category C or D, including patients with disseminated recurrent disease. Localized colorectal cancer corresponds to Dukes' A or B.

*11 ulcerative colitis, 13 diverticulitis, 15 ulcers, 9 primary biliary cirrhosis, 15 rectal polyps, and 5 gall bladder disease, etc.
References