Title: Titration of serum CEA, p53 antibodies and CEA-IgM complexes in 142 patients with colorectal cancer and 150 healthy blood donors.

Introduction

Colorectal cancers are the third most commonly diagnosed cancer and represent approximately 11% of all new cancer cases. Survival of patients with colorectal cancer depends largely on the stage of disease at diagnosis. In patients with localized disease, the five-year survival is approximately 90%, whereas in patients with regional spread, the five-year survival decreases to approximately 60%. In patients with distant metastases, the five-year survival is less than 10%.

Colonoscopy, an effective screening test, has been limited in its public acceptance due to its invasiveness and cost. Since measurement of tumor markers using serum, urine and feces is not invasive, the use of these tumor markers is expected to be more widely accepted for the screening of early colorectal cancers.

Although fecal occult-blood testing is widely used for noninvasive screening method, it has limited sensitivity: around 20%, while its specificity is about 95%. Carcinoembryonic antigen (CEA) in serum is also widely used. It was found to be elevated in up to 19% of smokers and in 3% of a healthy control population. It shows an overall sensitivity of 30–40% in discriminating healthy individuals from cancer patients. However, the value diminishes dramatically in cancer at early stage, dropping to less than 10% in Dukes’ Stage A colorectal cancer.

Recently, new type of serum marker is reported to overcome those limitations, for detecting early stage colorectal cancer. One example is serum p53 antibodies. The tumor suppressor p53 gene, located on chromosome 17p13.1, frequently undergoes mutation in the genesis of human cancer. The sensitivity of p53 antibodies in colorectal cancer is 24%, but it is higher in early stage than in advanced stage of colorectal cancer. The other example is CEA-IgM complexes. CEA-IgM complexes has overall sensitivity of 38% in colorectal cancer, and 29%
in early stage of colorectal cancer without compromising the detection specificity.

We have previously shown that, by combination of three tests, overall sensitivity was 53%, Stage I 31%, Stage II 53% and Stage III 79%, while false positive rate was 18%, by a single-institute small study of 67 patients\(^9\). Here we report the results of combination of above three tests in multi-institute larger study of 142 patients and 150 healthy donors.

**Patients and Methods**

**Patients**

142 patients with primary colorectal cancer who underwent surgery between June 2009 and July 2010 were enrolled in this study. This work was conducted as a multi-institutional study in 7 hospitals in Chubu Clinical Oncology Group. Written informed consent was obtained from each patient. No patients had received preoperative radiotherapy or chemotherapy. They were 77 (54%) male and 65 (46%) female patients, with an average age of 65.9 years (range, 40 – 91 years). The tumor was located in the colon in 93 cases (65%) and in the rectum in 49 cases (35%). Tumors were staged according to the International Tumor-Node-Metastasis (TNM) staging system and histological grade was assessed according to the World Health Organization (WHO) criteria. 37 of the tumors (26%) were Stage I, 34 (24%) were Stage II, 58 (41%) were Stage III, and 13 (9%) were Stage IV. 135 tumors (95%) were well differentiated, 7 (5%) were poorly differentiated. General information of patients is summarized in Table 1.

Serum samples from 150 healthy volunteers were analyzed as healthy control. Written informed consent was also obtained from these volunteers. They were 54 (36%) male and 96 (64%) female patients, with an average age of 41.3 years (range, 23 – 74 years).

**Serum samples**

Serum samples from patients were collected before surgery. All samples were stored at -80°C until they were assayed.

**Enzyme immunoassay for serum p53 antibodies**

Serum p53 antibodies levels were assessed by the anti-p53 EIA Kit II (MESACUP anti-p53 Test; Medical and Biological Laboratories, Nagoya, Japan). In brief, the samples were added, for one hour at room temperature, to microtiter wells coated with wild-type human p53 protein or a control protein to detect nonspecific interactions. After washing, a peroxidase-conjugated goat anti-human IgG that binds p53 antibodies was applied for one hour at room temperature. Then substrate solution was added for 30 min at room temperature. After the addition of stop solution, color development was assessed by measuring absorption at 450 nm, using photospectrometer. Levels of p53 antibodies were determined from a calibration curve constructed from the specific signals of standards. The cutoff value for serum p53 antibodies was 3.0 U/ml in this study.

**Enzyme immunoassay for serum CEA-IgM complexes**

Serum CEA-IgM complexes levels were assessed by the colon-IC ELISA Kit (Xeptagen, Pozzuoli, Italy). In brief, in 96 well ELISA plates, coated with anti-human CEA antibody, 100 µl of serially diluted reference standard or serum samples in dilution buffer were incubated for one hour at room temperature. After washing, the CEA-IgM complexes was incubated with enzyme-conjugated anti-human IgM for one hour at room temperature, and developed and assessed by measuring absorption at 405 m, using ELISA plate reader. Levels of CEA-IgM complexes were determined from a calibration curve constructed from the reference standard. The cutoff value was 150 AU/ml in this study.

**CEA assays**

Serum CEA concentrations were measured with Chemiluminescent Immuno assay, using ARCHITECT CEA (Abbott Japan, Tokyo, Japan). The cutoff value of CEA was 5 ng/ml according to the manufacturers.

**Statistical analysis**

The areas under the ROC curves were calculated using software R\(^{10}\).

**Results**

142 serum from patients suffering from colorectal cancers and 150 serum from non-colorectal cancer taken as control group were analyzed in parallel by CEA, p53 antibodies, CEA-IgM complexes assays.

The sensitivity of three tests is summarized in Table 2. In addition to overall patients, Stage I, II, III, IV and non-colorectal cancer (healthy control) are also described.

In Table 3, the combined determinations of three tests are shown. The diagnostic sensitivity with CEA and p53 antibodies was 48% (68/142), Stage I 16% (6/37), Stage II 56% (19/34), Stage III 53% (31/58), and Stage IV 92% (12/13), while false positive rate was 7% (10/150). Because the sensitivity of CEA-IgM was low, three tests combination did not much increase the overall sensitivity, considering the decrease of specificity.

Overlapping numbers of three tests were summarized in Table 4, showing a little overlapping of each test.

ROC curves were plotted for CEA, p53 antibodies and CEA-IgM complexes as shown in Fig. 1. The diagnostic accuracy measured as the areas under the ROC curves
showed that CEA is superior to p53 antibodies and CEA-IgM complexes (value: 0.888 vs 0.564 and 0.467).

**Discussion**

In the present study, the sensitivity of CEA-IgM for detecting colon cancer was lower than expected from previous study[6, 9], though it detected some Stage I patients which were not detected by CEA nor p53-antibodies (Table2-4). CEA-IgM has been already distributed for years in Europe and it had some results. The difference of sensitivity might be due to the racial difference of producing IgM, which is assumed to belong to natural antibody to cancer[11, 12], although the direct evidence has not been proved yet. For example, Japanese might have higher titer of natural antibody to CEA than European. Progressing another clinical study on CEA-IgM in Europe (AIGO study) would show some explanations for this.

The combination of CEA and p53-antibodies has fairly good detectability of colon cancer, especially in Stage II, III, IV. Of note, here, the cut-off level of p53-antibodies was set at 3.0 U/ml, instead of 1.3 U/ml according to the manufacturers. With cut-off at 1.3 U/ml, 26/150 healthy controls were false positive, decreasing the specificity at 83%. As it seemed to preserve sensitivity with cut-off at 3.0 U/ml, re-evaluation of cut-off level might be needed in p53-antibodies, which were recently approved by Ministry of Health, Labors and Welfare in Japan as tumor markers for esophageal cancer, breast cancer and colorectal cancer.

Apart from various approaches in new biotechnology for detecting new biomarker, such as fecal DNA panel[3] and SELDI-TOF-MS[13, 14], several new candidates for colon tumor marker have recently appeared such as MMP-9[15], PAI-1 or cathepsin[16] and dermokine[17], employing 'classic' ELISA method. As the chemotherapy to colon cancer has been multi-drug treatment[18], colon tumor marker of multi-form might be also needed. This concept was already shown by the combination of known tumor marker and various cytokines[9]. Though CEA-IgM did not show good results in this study, ‘biomolecule-IgM’ described by Fassina G, et al[20, 21] may be one of such new candidates.

<table>
<thead>
<tr>
<th>Histological classification</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
<th>healthy controls</th>
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<tbody>
<tr>
<td>Sample number</td>
<td>37</td>
<td>34</td>
<td>58</td>
<td>13</td>
<td>150</td>
</tr>
<tr>
<td>Male/Female</td>
<td>18/19</td>
<td>20/14</td>
<td>31/27</td>
<td>8/5</td>
<td>54/96</td>
</tr>
<tr>
<td>Age (mean)</td>
<td>40-85(66)</td>
<td>46-86(69)</td>
<td>35-91(64)</td>
<td>45-77(62)</td>
<td>23-74(41)</td>
</tr>
<tr>
<td>Colon/Rectum</td>
<td>25/12</td>
<td>25/9</td>
<td>36/22</td>
<td>7/6</td>
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<tr>
<td>Differentiated /Undifferentiated</td>
<td>36/1</td>
<td>33/1</td>
<td>53/5</td>
<td>13/0</td>
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Table 1. General information for the analyzed patients
### Table 2. Sensitivity of three tests

<table>
<thead>
<tr>
<th></th>
<th>Colorectal cancer</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall (n=142)</td>
<td>Stage I (n=37)</td>
</tr>
<tr>
<td>CEA positive</td>
<td>49 (35%)</td>
<td>2 (5%)</td>
</tr>
<tr>
<td>p53 antibodies</td>
<td>30 (21%)</td>
<td>4 (11%)</td>
</tr>
<tr>
<td>Complexes positive</td>
<td>13 (9%)</td>
<td>4 (11%)</td>
</tr>
</tbody>
</table>

*Cutoff value: CEA: 5 ng/ml, p53 antibodies: 3 U/ml, CEA-IgM complexes: 150 AU/ml

### Table 3. Sensitivity of three tests combination

<table>
<thead>
<tr>
<th></th>
<th>Colorectal cancer</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall (n=142)</td>
<td>Stage I (n=37)</td>
</tr>
<tr>
<td>CEA + p53 antibodies</td>
<td>68 (48%)</td>
<td>6 (16%)</td>
</tr>
<tr>
<td>CEA + CEA-IgM</td>
<td>57 (40%)</td>
<td>6 (16%)</td>
</tr>
<tr>
<td>CEA + p53 antibodies</td>
<td>74 (52%)</td>
<td>9 (24%)</td>
</tr>
</tbody>
</table>

*Cutoff value: CEA: 5 ng/ml, p53 antibodies: 3 U/ml, CEA-IgM complexes: 150 AU/ml

### Table 4. Overlapping numbers among three tests

(74 positive in 142 overall colorectal cancer)

<table>
<thead>
<tr>
<th></th>
<th>CEA also positive (n=49)</th>
<th>p53 antibodies also positive (n=30)</th>
<th>CEA-IgM complexes also positive (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA positive</td>
<td>-</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>p53 antibodies</td>
<td>11</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>CEA-IgM complexes</td>
<td>5</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>
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Acknowledgement
This work was supported by Chubu Clinical Oncology Group (CCOG) and Epidemiological and Clinical Research Information Network (ECRIN).

References

Fig 1. ROC curves comparing the distribution of serum levels of CEA, p53 antibodies and CEA-IgM complexes in patients with colorectal cancers (n=142) versus healthy control (n=150)