Titration of serum CEA, sFasL, CEA-IgM complexes and sFasL-IgM complexes in patients with gastric adenocarcinoma

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This work was supported by Chubu Clinical Oncology Group.
The samples were collected at Aichi Cancer Center, Aichi Hospital.
Conflicts of interest: No conflicts of interest exist.

Key words: gastric cancer, tumor marker, sFasL-IgM, immune complexes

Short title: For improvement of detecting gastric cancer in difficulty
Abstract

Background. The early detection of gastric cancer is important issue for improving the survival rate. As a serum marker, Carcinoembryonic antigen (CEA) and CA19-9 in serum is widely used. But their limited sensitivity is not suitable for screen method.

Methods. In this study, titer of CEA, sFasL, CEA-IgM and sFasL-IgM complexes were measured in serum from 86 patients with primary gastric cancer who underwent surgery. 48 of the tumors (56%) were Stage IA, 10(12%) were Stage IB, 6 (7%) were Stage II, 11 (13%) were Stage III, 11(13%) were Stage IV. Also, serum samples from 23 patients found to have no tumors with gastritis were analyzed as non-gastric cancer group.

Results. Overall, CEA-IgM was detected 20% (17/86), employing cut-off level with false positive rate 9% (2/23) in this cut-off level. sFasL-IgM was detected 12% (10/86), employing cut-off level with false positive rate 4% (1/23) in this cut-off level. CEA and sFasL detected advanced stages more than early stages, while CEA-IgM and sFasL-IgM detected early stages more than advanced stages. sFasL-IgM detected diffuse type more than intestinal type, while CEA-IgM intestinal type more than diffuse type. With 4 test, 40% (34/86) of overall gastric cancer, and 29% (14/48) of stageIA gastric cancer were detected.

Conclusion. sFasL-IgM are circulating in serum of early stages of diffuse type gastric adenocarcinoma. Employing multi-marker, as demonstrated here with 4 tests, might be needed, especially for gastric cancer which is hard to be detected by conventional serum marker.
**Introduction**

Gastric cancer is the most common malignancy of the gastrointestinal tract among the Japanese and Far Eastern populations and the second most common cancer worldwide (1). The outcome of patients with advanced or metastatic cancer remains poor, even after curative resection (2).

Early detection of gastric cancer by serum marker has long been a dream, which has not been accomplished yet. Since measurement of tumor markers using serum, is not invasive nor costing, the use of these tumor markers is expected to be more widely accepted for the screening of early gastric cancers.

One of the conventional tumor marker, CEA, is elevated in advanced stages of incurable cancer but very low (almost zero) in early stages. Another marker, CA19-9, is the same with sensitivity. The sensitivity of each marker is about 16%, low even in advanced stages, and they have, at most, prognostic value rather than diagnosis tool (3, 4).

Another is pepsinogen, which sensitivity is 55% with the specificity of 75%. But it has risk evaluation value rather than diagnostic tool (5, 6).

Recently, the elevation of sFasL in serum of gastric cancer was
reported (7). The rate of elevated sFasL level was 16%, 64.7%, 81.8%, 75% among patients with StageII, IIIA, IIIB, and IV gastric cancer, which are higher than conventional CEA and CA19-9, but sFasL hardly detected Stage IA and Stage IB.

On the other hand, we have detected CEA-IgM complexes in serum of gastric cancer, especially of intestinal type of early stages (8).

Here, we have measured CEA, sFasL, CEA-IgM complexes and sFasL-IgM complexes in patients with gastric adenocarcinoma. In addition, we show, for the first time, sFasL-IgM is circulating in serum of gastric cancer, especially of diffuse type of early stages.

**Patients and Methods**

**Patients**

86 patients with primary gastric cancer who underwent surgery at the Department of Gastroenterological Surgery, Aichi Cancer Center Aichi Hospital, Japan, between December 2005 and May 2007 were enrolled in this study. All operated patients were not enrolled. Written informed consent was obtained from each patient. No patients had received
preoperative radiotherapy or chemotherapy. They were 47 (55%) male and 39 (45%) female patients, with an average age of 66.0 years (range, 38 – 84 years). 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. 48 of the tumors (56%) were Stage IA, 10 (12%) were Stage IB, 6 (7%) were Stage II, 11 (12%) were Stage III, and 11 (12%) were Stage IV. 29 tumors (34%) were well differentiated, 57 (66%) were undifferentiated.

Serum samples from 23 patients underwent gastric endoscope examination and found to have no tumors with gastritis were analyzed as non-gastric cancer group (control). Written informed consent was also obtained from these patients. They were 9 (39%) male and 14 (61%) female patients, with an average age of 54.1 years (range, 40-71 years).

Patients’ background is summarized in Table 1.

**Serum samples**

Serum samples were collected from each patient before surgery or gastric endoscope examination. Samples were stored at -80°C until they were assayed.

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

We invented newly the system of sFasL-IgM detection. In brief, in 96 well ELISA plates, coated with anti-human sFasL antibody, 100µl of x5 diluted serum samples in dilution buffer were incubated for 24 hour at 4°C. After
washing, the sFasL-IgM complexes were incubated with enzyme-conjugated anti-human IgM for one hour at room temperature, and developed and assessed by measuring absorption at 405m, using ELISA plate reader. Levels of sFasL-IgM complexes were determined from a calibration curve constructed from the pooled serum. The cutoff value was set at 35 AU/ml with false positive rate 4% (1/23).

**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, 100µl 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 405m, using ELISA plate reader. Levels of CEA-IgM complexes were determined from a calibration curve constructed from the reference standard. The cutoff value was set at 120 AU/ml with false positive rate 9% (2/23).

**Enzyme immunoassay for serum CEA**

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

The level of sFasL in the serum was determined with an enzyme-liked immunoadsorbent assay (ELISA) kit for measuring sFasL (MBL, Nagoya, Japan). The cutoff value of sFasL was 0.1ng/ml according to the manufacturers.

**Results**

86 serum from patients suffering from gastric cancers and 23 serum from non-gastric cancer (with gastritis) taken as control group were analyzed in parallel by CEA, sFasL, CEA-IgM and sFasL-IgM complexes assays.

The results are summarized in Table2. Overall sensitivity is 5% (4/86), 6% (5/86), 20% (17/86) and 12% (10/86) in CEA, sFasL, CEA-IgM and sFasL-IgM assay, respectively. With 4 test, 40% (34/86) gastric cancer were detected.

CEA and sFasL detected no stageI cancer, but it detected advanced cancer, while CEA-IgM and sFasL-IgM detected early stages more than advanced stages (Table2).

In addition, sFasL-IgM detected diffuse type more than intestinal type, while CEA-IgM intestinal type more than diffuse type. As shown in Table3, in StageIA of diffuse type, sFasL-IgM was detected at 21% (6/28), while only 5% (1/20) of intestinal type. By employing both test, 29%
(14/48) was detected in StageIA gastric cancer, which is hardly detected by conventional serum tumor marker.

**Discussion**

Early detection of gastric cancer by serum marker has long been a dream, which has not been accomplished yet. Even in its advanced stages, the sensitivity is very low compared with that in colon cancer(9,10).

In order to overcome this situation, we re-tested sFasL, which might detect advanced stages of gastric cancer well (7). In our study, however, its sensitivity was not so high as already reported. Only 18% (5/28) of stageII-III-IV were detected by sFasL. Conventional CEA detected 14% (4/28). With both, since there was no overlapping, 32% (9/28) of stageII-III- IV were detected, while 0% (0/58) of stageI.

We have recently reported that CEA-IgM complex detected 20% of gastric cancer, 21% of stage I gastric cancer, and 36% of stageI gastric cancer of differentiated adenocarcinoma (8). It has characteristic that CEA-IgM detects early stages rather than advanced stages, as reported in case of colon cancer (9, 10).

In this report, we found that sFasL-IgM is, as well as CEA-IgM, elevated in gastric cancer.

Overall sensitivity is 20% (17/86) and 12 % (10/86) in CEA-IgM and sFasL-IgM assay. But in stage I gastric cancer, they detected 21% (12/58) and 14% (8/58) , respectively, and 31% (18/58) , by both.
As shown in Table 3, sFasL-IgM is likely to detect diffuse type more than intestinal type, as CEA-IgM intestinal type more than diffuse type (8).

The detection of sFasL-IgM in serum of gastric cancer is new in our report. We have tested sFas-IgM and DAF-IgM detection, which did not give good results (data not shown). It is supposed that “the secretion” from cancer cell is the condition necessary for the detection, because sFas nor DAF, unlike sFasL or CEA, may not be secreted according to the tumor progression in gastric cancer (11, 12).

The precise role of Fas/FasL system is still under discussion. By a so-called “counterattack model”, FasL-expressing tumor cells use FasL as a cytolytic effector molecule to kill Fas-expressing activated lymphocytes. This counterattack model suggests that the FasL may offer a survival advantage to tumors (13).

FasL expression was significantly higher in gastric carcinoma tissue samples, compared with normal gastric tissue samples, while the Fas expression level was significantly lower in gastric carcinoma tissue samples. And the FasL expression level was higher in diffuse type gastric adenocarcinoma than in intestinal type (14). Our observation that circulating sFasL or sFas-IgM exists more frequently in the diffuse type of gastric adenocarcinoma compared with intestinal type, may be consistent with this tissue expression rate.

The circulating immune complexes, “secreted protein from cancer cells and IgM immune complexes” are observed in colon cancer(9,10), HCC(15), prostate cancer(16), and other kinds of cancer (17).
One of the questions is why sFasL-IgM and CEA-IgM can be seen only in early stages and they seem to disappear in advanced stages.

One answer is that IgM has switched to IgG after recognition of cancer cell by T-cell. But we cannot detect sFasL-IgG nor CEA-IgG so far.

Other answer is that IgM in these immune complexes belongs to natural antibody to cancer cells (18). Some authors propose the existence of natural antibodies, instead of acquired antibodies, to cancer cells, which are restricted to the isotype IgM. According to them, the target of such IgM might be modified carbohydrate epitopes post-transcriptional in cancer cell. One of their example is anti-DAF IgM antibody which induces apoptosis of stomach carcinoma cells of, especially, diffuse type (12), though they do not mention on IgM immune complexes. The occupation of a lot of amount of free antigens may explain the difficulty of detecting immune complexes in advanced stages. Nevertheless, we don’t know if IgM in sFasL-IgM or CEA-IgM immune complexes induces apoptosis yet.

Here, we bring one previous observation to the hypothesis on the effect of natural IgM antibodies to cancer cells. It is reported that the reperfusion of autologous plasma perfused over immobilized proteinA lead to the necrosis of lesions in cancer patients (19). It is also reported later that proteinA is not necessarily needed, but the reperfusion of the extracorporal high salt-treatment or electric-treatment of autologous plasma is sufficient for inducing cancer necrosis in patients (personal communication). These treatments are common in making immune complexes in peace, proteins and IgM, and it is possible that this IgM separated from immune complexes
exert apoptosis in cancer patients in such phenomenon.

These are questions to be answered in future study of autoantibody to cancer cells (20).

Recently, new type of serum marker is reported to overcome the limitation of sensitivity, for detecting early stage gastric cancer.

One example is p53 autoantibody, which detected 15% of gastric cancer, and 10% of stage I gastric cancer (21). Other approach for detecting new biomarker is employed by SELDI-TOF-MS (22, 23). They reported good sensitivity and specificity, but its reproducitvity and availability (requiring SELDI-TOF-MS equipment) seems to be remained as problem.

Finally, as the chemotherapy to cancer has been multi-drug treatment, tumor marker of multi-form might be also needed (Figure). We hope that this report will be one start of such approaches, planning to test with larger population samples.

References


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Legends
Table 1 General information for the analyzed patients

Table 2 Sensitivity of CEA, sFasL, CEA-IgM and sFasL-IgM complexes titer (Overall patients)

Table 3 Sensitivity of CEA-IgM and sFasL-IgM complexes titer (Stage IA)

Figure The image of covering each marker in gastric cancer
Table 1: General information for the analyzed patients

<table>
<thead>
<tr>
<th>Histological classification</th>
<th>Stage IA</th>
<th>Stage IB</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
<th>gastritis</th>
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<tr>
<td>Sample number</td>
<td>48</td>
<td>10</td>
<td>6</td>
<td>11</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>Male/Female</td>
<td>total 47/39</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9/14</td>
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<td>Age (mean)</td>
<td>total 38-84 (66.0)</td>
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<td></td>
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<td>40-71 (54.1)</td>
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<td>Intestinal/diffuse</td>
<td>20/28</td>
<td>2/8</td>
<td>3/3</td>
<td>4/7</td>
<td>0/11</td>
<td>-</td>
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<tr>
<td>H. Pyrori status</td>
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<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>11 positive</td>
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<tr>
<td></td>
<td>Gastric cancer</td>
<td>control</td>
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<tr>
<td></td>
<td>Overall (n=86)</td>
<td>Stage I A (n=48)</td>
<td>Stage I B (n=10)</td>
<td>Stage I I (n=6)</td>
<td>Stage I II (n=11)</td>
<td>Stage I III (n=11)</td>
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<td>CEA positive</td>
<td>4 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (17%)</td>
<td>2 (18%)</td>
<td>1 (9%)</td>
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<tr>
<td>sFasL positive</td>
<td>5 (6%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3 (27%)</td>
<td>2 (18%)</td>
</tr>
<tr>
<td>CEA-IgM Positive</td>
<td>17 (20%)</td>
<td>9 (13%)</td>
<td>3 (30%)</td>
<td>0 (0%)</td>
<td>3 (27%)</td>
<td>2 (18%)</td>
</tr>
<tr>
<td>sFasL-IgM Positive</td>
<td>10 (12%)</td>
<td>7 (15%)</td>
<td>1 (10%)</td>
<td>1 (17%)</td>
<td>0 (0%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>Any positive</td>
<td>34 (40%)</td>
<td>14 (29%)</td>
<td>4 (40%)</td>
<td>2 (33%)</td>
<td>8 (72%)</td>
<td>6 (55%)</td>
</tr>
</tbody>
</table>

*cutoff value  
CEA: 5ng/ml, CEA-IgM complexes: 120AU/ml 
SfasL: 0.1ng/ml, SfasL-IgM complexes: 35AU/ml
# Table 3: Sensitivity of CEA-IgM and sFasL-IgM complexes titer (Stage IA)

<table>
<thead>
<tr>
<th></th>
<th>Stage IA Gastric cancer (n=48)</th>
<th>intestinal type (n=20)</th>
<th>diffuse type (n=28)</th>
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</thead>
<tbody>
<tr>
<td><strong>CEA-IgM</strong></td>
<td></td>
<td>6 (30%)</td>
<td>3 (11%)</td>
</tr>
<tr>
<td><strong>Positive</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>sFasL-IgM</strong></td>
<td></td>
<td>1 (5%)</td>
<td>6 (21%)</td>
</tr>
<tr>
<td><strong>positive</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CEA-IgM or sFasL-IgM positive</strong></td>
<td></td>
<td>6 (30%)</td>
<td>8 (29%)</td>
</tr>
</tbody>
</table>

* cutoff value: CEA-IgM complexes: 120AU/ml, sFasL-IgM complexes: 35AU/ml
Figure 1  The image of covering each marker in gastric cancer

Intestinal type

Diffuse type

early  Stage  advanced