Esophageal carcinoma frequently metastasizes to lymph nodes and directly invades neighboring major organs, including the lung, trachea, bronchi, and large vessels, often making complete tumor resection difficult. Concurrent preoperative chemoradiotherapy has been done to increase the resectability of advanced esophageal cancer (1–4), and its advantages are (a) increased resectability, (b) reduction of surgical stress caused by extensive resection of major organs, and (c) control of micrometastasis. Our previous study showed that preoperative chemoradiotherapy for stage III and IV squamous cell carcinoma of the esophagus significantly reduced postoperative recurrence and improved survival (4). However, preoperative chemoradiotherapy does not always provide these benefits in patients with advanced esophageal cancer. Retrospective analyses of resected specimens have revealed that survival was prolonged only when preoperative chemoradiotherapy was shown to be effective pathologically. We reported previously a statistically significant more unfavorable outcome in cases in which preoperative chemoradiotherapy was pathologically ineffective (grade 1) than in cases in which it was effective (grade 2 and 3; ref. 5), and similar results have been reported by other investigators (6–8). If preoperative chemoradiotherapy were not effective, the patients would not only receive no benefit from the preoperative chemoradiotherapy but also experience relatively severe side effects of preoperative chemoradiotherapy. They might also miss the chance for curative surgery because of metastasis that might occur during the 2-month period of preoperative chemoradiotherapy. Thus, there is an urgent need for new diagnostic modalities that can reliably predict the efficacy of preoperative chemoradiotherapy in advance.

The surface-enhanced laser desorption and ionization (SELDI) chip can uniformly capture, concentrate, and purify various complicated biological materials on its small chemical surface (9), which represents the most fundamental improvement of SELDI over matrix-assisted laser desorption and ionization (10). Comprehensive profiles of proteins captured
on SELDI chips can be created from samples of body fluid, such as serum, as small as a few microliters. SELDI-mass spectrometry (MS) analysis of the serum proteome has been reported to be capable of being used to diagnose malignancies of various organs, including the ovary, prostate, and pancreas (11–15), because serum protein profiles precisely reflect the micro-environmental conditions in diseased organs (16). This high diagnostic potential of SELDI-MS prompted us to investigate whether the proteomic profiles obtained from serum samples of untreated esophageal cancer patients could be used to predict the efficacy of preoperative chemoradiotherapy.

### Materials and Methods

#### Patients and serum samples.
Forty-two stage II to IV esophageal cancer patients treated with preoperative chemoradiotherapy and surgical resection at the Third Department of Surgery, Tokyo Medical University Hospital, between January 1998 and December 2001 were entered into this study. Preoperative chemoradiotherapy consisted of low-dose cisplatin (10 mg/m²/d, 5 days weekly for 2 weeks, biweekly, total 100 mg/m²), 5-fluorouracil (350 mg/m²/d, 5 days weekly for 4 weeks, total 7,000 mg/m²), and radiation (10-MV linear accelerator, 2 Gy/d, 5 days weekly for 4 weeks, total 40 Gy) administered concurrently. The histologic diagnosis of squamous cell carcinoma (Fig. 1).

#### Table 1. Clinical characteristics of 42 patients in this study

<table>
<thead>
<tr>
<th></th>
<th>Training set (n = 27)</th>
<th>Validation set (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responder (n = 15)</td>
<td>Nonresponder (n = 12)</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>61.7 ± 8.3</td>
<td>61.5 ± 5.27</td>
</tr>
<tr>
<td>Gender (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15 (100)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>Female</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tumor location 1 (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ce</td>
<td>0</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>Te</td>
<td>15 (100)</td>
<td>11 (91.7)</td>
</tr>
<tr>
<td>Ae</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clinical stage 2 (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>4 (26.7)</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>III</td>
<td>10 (66.7)</td>
<td>7 (58.3)</td>
</tr>
<tr>
<td>IV</td>
<td>1 (6.7)</td>
<td>3 (25)</td>
</tr>
<tr>
<td></td>
<td>Responders (n = 10)</td>
<td>Nonresponders (n = 5)</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>62.2 ± 12.0</td>
<td>62.5 ± 4.17</td>
</tr>
</tbody>
</table>

* Analyzed using Student’s t test.
† Analyzed using Fisher’s exact t test.
Tumor location and clinical stage were classified according to the Guidelines for Clinical and Pathologic Studies on Carcinoma of the Esophagus (17).
To denature serum proteins, 10 A to pH 9 was added to 10

The study was reviewed and approved by the Ethics

Table 2. Reproducibility of 859 mass peaks detected by SELDI-QqTOF-MS

<table>
<thead>
<tr>
<th>Array/wash condition</th>
<th>No. detectable peaks</th>
<th>Correlation of coefficient (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H50</td>
<td>235</td>
<td>0.980 ± 0.016</td>
</tr>
<tr>
<td>CM10/pH 4</td>
<td>207</td>
<td>0.955 ± 0.040</td>
</tr>
<tr>
<td>CM10/pH 7</td>
<td>181</td>
<td>0.972 ± 0.030</td>
</tr>
<tr>
<td>Immobilized metal affinity capture coupled with copper</td>
<td>236</td>
<td>0.942 ± 0.079</td>
</tr>
<tr>
<td>Total</td>
<td>859</td>
<td>0.960 ± 0.019</td>
</tr>
</tbody>
</table>

was confirmed in endoscopic biopsy specimens before preoperative chemoradiotherapy, and surgical resection was done ~ 4 weeks after the completion of preoperative chemoradiotherapy. The effects of preoperative chemoradiotherapy were classified into grade 0 (ineffective), grade 1 (slightly effective; Fig. 1A and C), grade 2 (moderately effective), and grade 3 (markedly effective; Fig. 1B and D) according to the criteria described previously (ref. 17; Supplementary Material 1). Informed consent was obtained from all patients. Blood samples were collected 3 to 5 days before preoperative chemoradiotherapy and allowed to coagulate at room temperature. Serum was obtained by centrifugation at 3,000 rpm for 30 minutes and cryopreserved at -80°C until analyzed.

Surface-enhanced laser desorption and ionization/hybrid quadrupole time-of-flight mass spectrometry analysis. To denature serum proteins, 90 µL U9 buffer [9 mol/L urea, 2% CHAPS, and 50 mmol/L Tris-HCl (pH 9)] was added to 10 µL of each sample and vortexed for 20 minutes. To increase the number of detectable protein peaks, we used four different ProteinChip (Ciphergen Biosystems, Inc., Fremont, CA) arrays coupled with copper (IMAC-Cu2+). Notched peaks appeared in H50, CM10/pH 7, CM10/pH 4, and immobilized metal affinity capture coupled with copper (IMAC-Cu2+) arrays. Note that >90% of the ionized protein peaks are plotted within 2-fold differences.

Fig. 3. Distribution pattern of the 7,420 m/z peak. Average intensity (average of duplicate measurements in arbitrary units) of the 7,420 m/z peak in the 42 serum samples of the training set (left) and validation set (right). The difference in distribution of intensity between nonresponders (Non-Res.) and responders (Res.) in both sets was significant (Mann-Whitney U test). The distribution of all other peaks is available in Supplementary Material 2.

array/wash conditions i.e., reversed phase (H50), weak cation exchanger with low stringent wash (CM10/pH 4), cation exchanger with high stringent wash (CM10/pH 7), and immobilized metal affinity capture coupled with copper as instructed by the supplier. Each sample was randomly assigned in duplicate to 1 of 96 spots of 12 allied ProteinChip arrays with a Biomex 2000 laboratory workstation (Beckman Coulter, Inc., Fullerton, CA). Sinapinic acid solution was prepared in 50% (v/v) acetonitrile and 5% (v/v) trifluoroacetic acid as an energy-absorbing matrix, and saturated solution (1 µL) was applied to each spot on the chips.

Low molecular weight proteins in the 2,000 to 40,000 m/z range were read on a high-resolution performance hybrid quadrupole time-of-flight MS (QqTOF-MS) Q-star XL (Applied Biosystems, Foster City, CA) equipped with a PCI 1000 ion source (Ciphergen Biosystems). The laser intensity, frequency, and accumulation time of the instrument were set at 60%, 25 Hz, and 90 seconds, respectively. Mass accuracy was externally calibrated on the day of the measurements by using the all-in-one-peptide molecular mass standard (Ciphergen Biosystems).

Peak detection and quantification. The mass data were converted to text files that consist of m/z and intensity after mass calibration by Analyst QS (Applied Biosystems) and processed by the procedures described in Supplementary Material 1 (18). Peak images were generated with Analyst QS and MassNavigator (Mitsui Knowledge Industry Co. Ltd., Tokyo, Japan) software.

Statistical analysis. Statistical differences were evaluated by Fisher’s exact probability t test, Student’s t test, and Mann-Whitney U test. We did binary classification using the support vector machine (SVM) algorithm such that the distance between the closest training example (the support vector) and the separating hyperplane would be maximized in a much higher dimensionality feature space transformed from the original input space with a kernel function (19). We used SVM with the Gaussian kernel to search to discriminate combinations of peaks. The SVM distance was calculated from the formula described in Supplementary Material 1.

Fig. 2. Correlation analysis of quantitative data obtained from a serum sample. Two-dimensional plot of mass intensities of corresponding duplicated peaks that appeared in H50, CM10/pH 7, CM10/pH 4, and immobilized metal affinity capture coupled with copper (IMAC-Cu2+) arrays. Note that >90% of the ionized protein peaks are plotted within 2-fold differences.
Results

Serum protein profiling by surface-enhanced laser desorption and ionization-hybrid quadrupole time-of-flight mass spectrometry. The clinicopathologic characteristics of the 42 patients enrolled in this study are summarized in the Table 1. We defined cases with the grade 2 or 3 effects in response to preoperative chemoradiotherapy as responders and cases with the grade 1 effects as nonresponders, because there was a significant difference in outcome between these two groups (5). No cases were classified as grade 0. Serum samples were obtained before preoperative chemoradiotherapy with informed consent, and they were stored frozen until the SELDI-QqTOF-MS analysis. Peaks were detected and quantified from raw mass spectra by using custom algorithms specially designed for SELDI-QqTOF-MS (described in Materials and Methods).

It was possible to extract a total of 859 mass peaks that were allied all across 42 duplicate serum samples in the 2,000 to 40,000 m/z range (Table 2). Mass deviation was within 0.05% throughout the study. The average intensities of the duplicate samples for these 859 mass peaks were used for the subsequent analyses. The mean correlation coefficient of 859 peaks between the duplicates of the 42 serum samples was 0.960 ± 0.019 (0.942-0.980; Table 2; Fig. 2).

Selection of a candidate classifier in the training set by machine learning. We selected 15 responders and 12 nonresponders with no statistically significant difference in age, sex, tumor location, or clinical stage as the training set from the total of 42 cases (Table 1). The remaining 15 cases were maintained apart as a blinded validation set. Based on the 27 samples in the training set, we estimated that not more than five markers would be necessary to achieve 100% discrimination between responders and nonresponders.

Fig. 4. Representative mass spectra showing the four peaks of the classifier at 7,420 (H50), 9,112 (H50), 17,123 (CM10/pH 4), and 12,867 (immobilized metal affinity capture coupled with copper) m/z in a nonresponder (NT06) and a responder (RT01). Arrows and numbers, classifier peaks and their relative intensities (in arbitrary units).
responders and nonresponders based on recursive feature elimination (ref. 20; data not shown). Because it is impossible to check all possible combinations of five markers in time, we first selected a peak at 7,420 m/z (H50) that was the most significantly different between responders and nonresponders in the training set ($P = 0.0087$, Mann-Whitney $U$ test; Fig. 3). We then searched for supplementary peaks step-by-step until 100% selection of responders was achieved. Although marker sets that do not contain 7,420 m/z may be overlooked by this strategy, the probability of that occurring was thought to be low despite the significant reduction of labor. We found that a minimal set consisting of four peaks at 7,420 (H50), 9,112 (H50), 17,123 (CM10/pH 4), and 12,867 (immobilized metal affinity capture coupled with copper) m/z (Fig. 4) diagnosed 15 responders in the training set with 100% specificity.

The selection of the set of four peaks (i.e., the classifier) was evaluated by leave-one-out cross-validation. The classifier was able to distinguish the responders in the training set with 100% (15 of 15) sensitivity and the nonresponders with 91.7% (11 of 11), a negative predictive value of 100% (4 of 4), and an overall accuracy of 93.3% (14 of 15).

**Discussion**

Comparative proteomic profiling coupled with computerized machine learning without actual identification of the specific proteins seems to be a rapid and promising alternative method to traditional single protein assays. Multivariate analyses using proteomic data obtained by SELDI-MS have been reported to be highly successful for serum detection of malignancies of various organs (11–16), and this approach may revolutionize medical practice and cancer diagnosis. Resistance to chemotherapy and radiotherapy is probably mediated by a variety of molecular pathways, and it seems reasonable to assume that it is impossible to predict sensitivity to chemotherapy and radiotherapy by measuring any single biomarker. However, it should be borne in mind that multivariate discrimination is dependent on stacks of small differences between cases and controls and must always be corroborated by high accuracy of each measurement. To improve mass accuracy and resolution of MS measurements and minimize day-to-day and machine-to-machine drift, we used a high-resolution QqTOF-MS instrument. Our quality-control experiments revealed that the correlation coefficients between three independent measurements of a pool of plasma samples done every other day were 0.972 to 0.992 (data not shown). The advantage of high-resolution machines was originally predicted by Petricoin and Liotta (16). They divided raw mass spectra into 7,084 “bins” with identical spaces and a fixed m/z, whereas we developed custom algorithms to detect and align peaks in a rather conventional way and searched for markers using SVM and leave-one-out cross-validation rather than a genetic algorithm. The number of detectable peaks reached 859 per sample, thanks to the high-resolution mass separation. High reproducibility and low day-to-day mass variation (<0.05%) were achieved by using this high-resolution instrument in this study. We will describe our data for the comparison between low-resolution and high-resolution instruments elsewhere (21).

One nonresponder was misclassified as a responder (Fig. 5). We tried to select the classifier that would identify 100% of the responders with the smallest percentage of misdiagnosis of nonresponders. Predicting responders who are actually nonresponders is more problematic than predicting nonresponders who are responders, because the former type of misclassification may deprive patients whose tumors are sensitive to preoperative chemoradiotherapy of the benefit of preoperative chemoradiotherapy.

Baggery et al. (22) cautioned about the reproducibility of conventional SELDI-MS analysis. In this study, we selected a classifier consisting of four peaks that distinguished responders to preoperative chemoradiotherapy from nonresponders in the training set. None of the four peaks were in the <2,000 m/z range, where chemical noise derived from the matrix often hamper correct measurement (22). We also confirmed that they

![Fig. 5. Classification by the fixed discriminating hyperplane. Scatter graph showing the SVM distance of 42 cases in the training set and validation set. Cases with positive distances were diagnosed as “responders” and cases with negative distances were diagnosed as “nonresponders.” NT, nonresponders in the training set; RT, responders in the training set; NV, nonresponders in the validation set; RV, responders in the validation set.](image-url)
were not derived from mass noise by visual inspection (Fig. 4). Most errors in biological measurements occur because of preanalytic bias derived from variables in the sample population as well as the sample collection, handling, and processing procedures (22, 23). We examined the serum samples of all consecutive patients with advanced esophageal cancer treated during a certain period and matched various clinical variables of the cases to be compared (Table 1). All serum samples were obtained from untreated patients and processed in the same manner without knowing the results of preoperative chemoradiotherapy; thus, preanalytic bias is unlikely in our study.

There are pros and cons to SELDI-MS with high-resolution QqTOF instruments. One of the two limitations of this study is the unavailability of protein identification. QqTOF-MS can detect peaks with one-digit or two-digit smaller intensities than low-resolution TOF-MS. The intensity of many peaks detected by SELDI-QqTOF-MS seems to be below the sensitivity of tandem MS. Furthermore, it was difficult to purify proteins from these low-intensity peaks without contamination by neighboring high-intensity peaks. Fourier transform MS may overcome these problems, because it does not depend on enzymatic digestion as a precondition for protein identification (24). However, no interface to the SELDI chip is currently available for Fourier transform MS. Many low molecular weight proteins detected by SELDI-MS in serum samples have been reported to be metabolic products, proteolytic fragments, cytokines, and peptides produced as a result of the host-tumor interaction in the microenvironment of the tumor (16, 25, 26). Shimada et al. (27) reported that expression of angiogenic factors predicted the response of esophageal cancer to chemoradiotherapy. We may have to consider the possibility that host factors have an effect on the chemoradiosensitivity of cancer. Actually, an active host reaction is often seen after killing of cancer cells by chemoradiotherapy as shown in Fig. 1D. The second limitation is the number of cases examined. It would be not easy to obtain a larger series of advanced esophageal cancers treated with preoperative chemoradiotherapy at one institution. However, the novel findings in this study warrant a large-scale multi-institutional analysis to confirm the feasibility of serum proteomic profiling as a means of predicting sensitivity to chemotherapy and radiotherapy.

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References

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