ProteinChip Array Profiling for Identification of Disease- and Chemotherapy-Associated Biomarkers of Nasopharyngeal Carcinoma

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Background: We previously used ProteinChip array profiling analysis to discover a serum biomarker associated with nasopharyngeal carcinoma (NPC). In this study, we used the same method to examine other biomarkers associated with NPC and response to chemotherapy (CT) in NPC patients.

Methods: We performed ProteinChip array analysis in 209 serum samples from 66 relapsed patients before and after salvage CT with gemcitabine and cisplatin or etoposide and cisplatin combinations, 11 patients in remission, and 35 healthy individuals. Intensities of the biomarker peaks were correlated with CT response of the patients and other clinical parameters.

Results: We discovered 13 candidate biomarkers associated with different clinical parameters. Two biomarkers (2803 and 3953 Da) were significantly increased in patients compared with controls at all stages of disease. Analysis of pre- and post-CT paired serum samples revealed 7 biomarkers correlated with impact of CT. Of these 7 biomarkers, 2 (2509 and 2756 Da) were significantly increased and 5 (7588, 7659, 7765, 7843, and 8372 Da) were significantly decreased post-CT in either 1 or both CT cohorts. Four biomarkers from pre-CT sera were correlated with CT response, with 3 (2950, 13 510, and 14 855 Da) being significantly decreased and 1 (6701 Da) significantly increased in patients who did not respond to CT. Tandem mass spectrometric sequencing and/or immunoaffinity capture assay identified the 3953 Da biomarker as a fragment of inter-alpha-trypsin inhibitor precursor and 7765 Da biomarker as platelet factor-4.

Conclusions: Treatment-associated serum biomarkers found might serve to triage NPC patients for appropriate CT treatment.

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Nasopharyngeal carcinoma (NPC)3 is the 6th most prevalent cancer among the male population in Hong Kong, (1) and is one of the most common cancers in Southern China and South East Asia. This malignancy occurs sporadically in the west but has a relatively high annual incidence rate of 15 to 50 per 100 000 in Southern China. Although NPC is highly sensitive to radiotherapy (RT) and chemotherapy (CT), posttreatment relapse often occurs. A retrospective analysis of >5000 NPC patients treated in Queen Elizabeth Hospital Hong Kong found that although complete response to RT occurred in 83% of patients, 53% of patients finally developed locoregional or distant relapse at a mean period of 1.4 years posttreatment (2). Even with state-of-the-art RT and radiological staging, 5-year progression-free survival is only 63% (3). Patients who suffer locoregional or distant relapse are often treated with salvage CT, but even after CT, >98% of relapsed patients die of disease (4). The emergence of drug resistance may be a major reason for such poor survival (5), and the role
of CT in salvaging patients with recurrent or metastatic NPC must be further evaluated. Biomarkers associated with treatment response might serve as useful indicators to triage patients for appropriate CT treatment.

We previously used ProteinChip array profiling to discover that the levels of serum amyloid A protein (SAA) are increased at the time of relapse in NPC patients (6). In the present study, we used the same method to identify treatment- or chemoresponse-associated serum biomarkers in relapsed NPC patients recruited into 2 phase II CT trials of salvage treatment with combination etoposide and cisplatin (EP cohort) and combination gemcitabine and cisplatin (GC cohort).

**Materials and Methods**

**NPC patients and treatment protocols**

This study recruited 77 NPC patients who received primary external megavoltage RT with curative intent. Among them, 11 patients had complete remission of disease for >8 years after RT and 66 patients suffered from either locoregional recurrences (LRR) or distant metastases (DM) after RT. The relapsed patients were entered into 1 of 2 prospective phase II clinical trials of salvage CT in which they underwent treatment with either EP (7) or GC regimen (8). Both clinical trials were approved by the Queen Elizabeth Hospital Ethics Committee and were conducted in accordance with the Helsinki Declaration. Informed written consents were obtained from all patients before enrollment. The EP cohort comprised 35 patients (male:female ratio of 32:3; age distribution of 30–65 years; mean age, 48.4 years) treated at relapse; among them, 1 patient was at UICC (International Union Against Cancer) stage II, 13 in stage III, and 19 in stage IV (2 had no staging information). After primary RT, 5 patients developed LRR, 7 developed DM in a single site, 16 developed DM with or without LRR in 2 sites, and 7 developed DM in >2 sites. The ambulatory CT scheme consisted of cisplatin at 35 mg/m² and etoposide at 100 mg/m² for 3 days in each cycle. After EP CT, 15 patients achieved complete or partial response (chemoresponders, RS) and 20 patients had either static or progressive disease (nonresponders, NR) classified according to WHO criteria. Another 31 patients at relapse (male:female ratio 28:3; age distribution of 31–64 years, mean age 48.1 years) were treated with the GC regimen, the cohort comprised 4 patients in Ho’s stage II, 20 in stage III, 6 in stage IV, and 1 in stage V. After primary RT, 7 patients developed LRR, 9 developed DM in a single site, 7 developed DM with or without LRR in 2 sites, and 8 developed DM in >2 sites. Salvage CT was applied to the relapsed patients with gemcitabine administered at a dose of 1000 mg/m² and cisplatin given at a dose of 50 mg/m² on 2 separate days in each cycle. There were a total of 24 chemoresponders and 5 nonresponders after CT (2 patients, whose responses were not evaluable, were excluded from the analysis).

**SERUM SAMPLES**

We collected 174 blood samples from 11 NPC patients in remission and 66 patients in relapse. In the EP cohort, 35 samples were obtained at the time of relapse before salvage CT and another 35 collected within an average of 22 days (at a range of 0–139 days) after CT. In the GC cohort, 20 blood samples were collected at initial diagnosis, 31 at relapse pre-CT, and 31 post-CT within an average of 13 days after CT (range, 0–90 days). For the remission patients, 11 blood samples were obtained at initial diagnosis and another 11 at various time points during follow-up while in remission after RT. We obtained single blood sample from each of the 35 healthy individuals (sex and age were known for 29 out of 35 individuals; male:female ratio, 12:17; mean age 34.1 years; 6 sera were from anonymous healthy donors). From each sample, 8 mL blood was allowed to clot at 4 °C for at least 2 h and then centrifuged at 1500g for 10 min to sediment the clotted cells. Sera were collected, divided into aliquots, and stored frozen at −70 °C until ProteinChip array profiling analysis was carried out.

**PROTEINCHIP PROFILING ANALYSIS**

Serum proteins/peptides from NPC patients and controls were first bound onto Q Ceramic HyperD F anion exchange beads (Ciphergen Biosystems, USA) and eluted into 5 fractions with aqueous buffers at pH 9, 7, 5, 4, and 3, and finally with an organic buffer as described in our previous paper (6) and in a book chapter from our team (9). Each fraction was profiled on a Copper (II) Immobilized Metal Affinity Capture (IMAC3-Cu [III]) ProteinChip® Array (Ciphergen Biosystems) according to the manufacturer protocols (10). The arrays were analyzed in a Protein Biological System Iic (PBS-Iic) mass spectrometer (Ciphergen Biosystems).

**DATA AND STATISTICAL ANALYSES**

ProteinChip profiling spectra were generated from each serum fraction with proteins/peptides displayed as unique peaks based on their mass-to-charge ratio (m/z) as analyzed by Ciphergen ProteinChip Software 3.0.2. Each peak was first baseline subtracted, then normalized with mean total ion current and included for analysis with a cutoff signal-to-noise ratio >5 for the 1st pass and >2 for the 2nd. Two biomarker peaks were defined as distinctly different by a preset mass difference tolerance of 0.3%. All 13 biomarker peaks in Table 1 were discovered from pH 9 fractions and, according to the Mann–Whitney U-test, had the greatest significant differences between analyzed subgroups. Using a Classification and Regression Tree algorithm from Biomarker Pattern Software, we combined 2 biomarkers (2950 and 6701 Da) for classification tree analyses between CT responders and nonresponders (11) with a cutoff intensity of 1.571 for peak 2950 Da in node 1 and 1.596 for peak 6701 Da in node 2. The CVs in peak intensities were previously reported in our book chapter to be 8%–26% (9). Owing to the restricted number of
Table 1. Overview of biomarkers associated with various clinical parameters in NPC patients.

<table>
<thead>
<tr>
<th>Patient groups compared</th>
<th>MW, Da</th>
<th>Test performed</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Accuracy, %</th>
<th>Increase or decrease</th>
<th>Diff, %</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) Cancer associated biomarkers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP vs Normal</td>
<td>2803</td>
<td>Mann-Whitney U-test</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>Inc in NPC</td>
<td>2086</td>
<td>1.0E-10</td>
</tr>
<tr>
<td>DX vs Normal</td>
<td>2803</td>
<td>&quot;</td>
<td>96</td>
<td>94</td>
<td>95</td>
<td>&quot;</td>
<td>907</td>
<td>1.0E-10</td>
</tr>
<tr>
<td>REM vs Normal</td>
<td>2803</td>
<td>&quot;</td>
<td>94</td>
<td>97</td>
<td>96</td>
<td>&quot;</td>
<td>864</td>
<td>1.3E-7</td>
</tr>
<tr>
<td></td>
<td>3953</td>
<td>&quot;</td>
<td>100</td>
<td>94</td>
<td>97</td>
<td>&quot;</td>
<td>700</td>
<td>1.0E-10</td>
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<tr>
<td><strong>(B) Treatment associated biomarkers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PostCT vs Pre-CT</td>
<td>2509</td>
<td>&quot;</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Inc in Post-CT</td>
<td>389</td>
<td>6.3E-3</td>
</tr>
<tr>
<td>EP cohort</td>
<td>7588</td>
<td>&quot;</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Dec in Post-CT</td>
<td>60</td>
<td>1.8E-4</td>
</tr>
<tr>
<td></td>
<td>7659</td>
<td>&quot;</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>&quot;</td>
<td>48</td>
<td>4.8E-6</td>
</tr>
<tr>
<td></td>
<td>7765</td>
<td>&quot;</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>&quot;</td>
<td>53</td>
<td>3.2E-4</td>
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<tr>
<td></td>
<td>8372</td>
<td>&quot;</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>&quot;</td>
<td>49</td>
<td>6.7E-6</td>
</tr>
<tr>
<td>PostCT vs Pre-CT</td>
<td>2756</td>
<td>&quot;</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Inc in Post-CT</td>
<td>179</td>
<td>6.2E-3</td>
</tr>
<tr>
<td>GC cohort</td>
<td>7659</td>
<td>&quot;</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Dec in Post-CT</td>
<td>50</td>
<td>1.1E-4</td>
</tr>
<tr>
<td></td>
<td>7765</td>
<td>&quot;</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>&quot;</td>
<td>51</td>
<td>6.1E-6</td>
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<tr>
<td></td>
<td>8343</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>&quot;</td>
<td>49</td>
<td>1.4E-5</td>
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<td><strong>(C) Chemo-response associated biomarkers</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NR vs RS</td>
<td>2950</td>
<td>&quot;</td>
<td>90</td>
<td>53</td>
<td>74</td>
<td>Inc in RS</td>
<td>182</td>
<td>3.9E-2</td>
</tr>
<tr>
<td>EP cohort</td>
<td>6701</td>
<td>&quot;</td>
<td>75</td>
<td>67</td>
<td>71</td>
<td>Inc in NR</td>
<td>131</td>
<td>4.9E-2</td>
</tr>
<tr>
<td></td>
<td>2950–6701</td>
<td>Node/tree analysis</td>
<td>80</td>
<td>87</td>
<td>83</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>NR vs RS</td>
<td>13 510</td>
<td>Mann-Whitney U-test</td>
<td>60</td>
<td>100</td>
<td>93</td>
<td>Inc in RS</td>
<td>181</td>
<td>7.9E-3</td>
</tr>
<tr>
<td>GC cohort</td>
<td>14 855</td>
<td>&quot;</td>
<td>60</td>
<td>96</td>
<td>90</td>
<td>&quot;</td>
<td>183</td>
<td>2.4E-2</td>
</tr>
</tbody>
</table>

CT, chemotherapy; Dec, decrease; DX, nasopharyngeal carcinoma at diagnosis; EP cohort, cohort of patients treated by chemotherapy with Etoposide and Cisplatinum for relapse; GC cohort, cohort of patients treated by chemotherapy with Gemcitabine & Cisplatinum for relapse; Inc, increase; NA, not applicable; NPC, nasopharyngeal carcinoma; NR, nonresponders in chemotherapy; REM, nasopharyngeal carcinoma patients in remission; RP, nasopharyngeal carcinoma patients in relapse; RS, responders in chemotherapy. (Diff, %), percentage difference of peak intensity was calculated in (A). Cancer-associated biomarkers by dividing the average peak intensity (API) of each biomarker in patients at relapse, initial diagnosis, or remission by that of the normal controls and multiplying by 100%; in (B). Treatment-associated biomarkers by dividing API of each biomarker in the posttreatment sera by that in the pretreatment sera and multiplying by 100%; in (C). Chemoresponse-associated biomarkers by dividing API of each biomarker in the chemoresponders by that in the nonresponders and multiplying by 100%. All 13 biomarker peaks reported in this table were discovered in fraction 1 of the ion exchange bead fractionated sera by elution at pH 9.
patients recruited in the 2 CT trials, there were differences from controls in sex ratio and mean age. When we compared the intensities of 2 representative biomarkers at 2803 and 3953 Da stratified by sex and age (mean age of the youngest vs oldest 10 controls, 21.4 vs 46.8 years), we did not observe any significant differences. This finding illustrated that the differences in peak biomarker intensities found in the patients are unlikely to be due to sex or age differences.

PROTEIN IDENTIFICATION

Direct Sequencing of the 3953-da Biomarker. The PH9 fraction was bound to IMAC30-Cu(II) ProteinChip Arrays, and the bound proteins were first analyzed with a QSTAR XL tandem mass spectrometer (Applied Biosystems/MDS Scieix) equipped with PCI-1000 ProteinChip Interface (Ciphergen Biosystems) in a single mass spectrometry (MS) mode. The 3953-Da peptide was detected as an ion with monoisotopic m/z of 3953.88. This ion was fragmented by collision-induced dissociation, and the resulting MS/MS data were submitted to the database-mining tool Mascot (Matrix Sciences) for protein identification. The following identification search parameters were used: Database-SwissProt, taxonomy-human (14 445 entries), enzyme–none, variable modifications–pyro-Glu, peptide tolerance–20 ppm, MS/MS tolerance–0.2 Da.

Immunoaffinity Capture by Anti-ITIH4 and Anti-PF4 Antibodies. We precoated 1 µL of anti-ITIH4 monoclonal antibody (1 g/L, Ciphergen Biosystems) onto a RS100 ProteinChip array (Ciphergen Biosystems). After blocking with bovine serum albumin and Tween-20 and washing, 1 µL of concentrated serum fraction was reacted on the antibody-precoated array for 4 h at 4 °C with shaking. The array was washed twice with buffer containing urea and Tween followed by water once. After washing, the array was air-dried and 1 µL cyano-4-hydroxycinnamic acid was added twice. The antibody-captured proteins were profiled on the PBS-II mass spectrometer as previously described. Immunoaffinity capture by a polyclonal anti-PF4 antibody (0.2 g/L, Chemicon) was similarly performed.

Results
We previously used ProteinChip array profiling technology to identify a serum biomarker, SAA, which could be useful in monitoring relapse in NPC patients (6). In the present study, we discovered 13 biomarkers associated with various clinical features in the patients undergoing treatment in CT trials, a GC cohort, and an EP cohort (Table 1), as described below.

BIOMARKERS ASSOCIATED WITH NPC

Confirming our previous study, SAA was significantly increased by 4.7-fold in the relapsed patients of the present study (data not shown). Two newly discovered serum biomarkers at 2803 and 3953 Da were also significantly increased in mean peak intensities in relapsed patients vs controls (Figs. 1A and B). Unlike SAA, the 2 new biomarkers were significantly increased in patients compared with controls also at initial diagnosis and in remission although, at lower peak intensities (Fig. 1C).

BIOMARKERS ASSOCIATED WITH THE IMPACT OF SALVAGE CT

Biomarkers correlating with the impact of CT were subsequently investigated. Using paired pre- and post-CT sera from relapsed patients in the 2 CT cohorts, we discovered significant alterations of average peak intensities in 7 biomarkers after CT (Fig. 2 and Table 1). For instance, a biomarker at 2509 Da was significantly increased in mean peak intensity after CT in the EP cohort, in sharp contrast to the other 4 biomarkers at 7588, 7659, 7765, and 8372 Da in the same cohort, which had significantly decreased post-CT peak intensities. Four biomarkers with significant peak intensity alterations post-CT were also found in the GC cohort. The post-CT serum peak intensities of the biomarker at 2756 Da were significantly increased. This biomarker also differed distinctly from the other 3 biomarkers at 7659, 7765, and 7843 Da, which had significant decrease in the post-CT sera of the GC cohort. Among all these marker peaks, 2 biomarkers at 7659 and 7765 Da had a similar trend of alteration in both cohorts. Alterations in the other 3 biomarkers (2509, 7588, and 8372 Da) appeared to be restricted only to the EP cohort, whereas changes in 2 biomarkers (2756 and 7843 Da) were confined to the GC cohort alone.
Protein Identities of 2 Biomarkers

3953 Da Peptide is ITIH4. The 3953 Da peptide was identified by direct sequencing with a Q-STAR XL tandem mass spectrometer equipped with PCI-1000 ProteinChip Interface. Analysis of MS/MS spectra with the Mascot mining tool and the SwissProt human database resulted in identification of only 1 peptide, with the amino acid sequence: QAGAAGSRMNFRPGVLSSRQLGLPGPDVPDHAAYHPF and with a Mowse score of 21, which is slightly lower than that for significant homology (a score $>36$) (Fig. 4A). The theoretical m/z value for the peptide was calculated to be 3953.95, and the experimentally observed value was 3953.88 (18 ppm error). The identified peptide corresponds to the fragment of human

Fig. 1. Cancer-associated serum biomarkers in nasopharyngeal carcinoma patients.

(A), Biomarker: 2803 Da. The normalized peak intensities of a biomarker at 2803 Da in NPC patients at relapse (RP), initial diagnosis (DX), and remission (REM) were all significantly increased compared with those from the control individuals (NM). (B), Biomarker: 3953 Da. A similar increase in NPC patients vs controls of the normalized peak intensities in a 2nd biomarker at 3953 Da in the same clinical subgroups. (C), Spectral views – 2803 and 3953 Da. Spectra of the 2 biomarkers, 2803 and 3953 Da in patients in different clinical subgroups as mentioned above were illustrated.
intero-trypsin inhibitor heavy chain H4 precursor (ITIH-4, SwissProt #Q14624). This identity was confirmed by ProteinChip immunoaffinity capture assay with anti-ITIH-4 antibody, which specifically captured the 3953 Da peptide in serum fractions of 3 NPC patients (NPC1, 2 and 3) but not or in only very small quantity in the 3 control serum fractions (Fig. 4B), concurring with the initial biomarker discovery results (Fig. 1). Because the identified peptide contains methionine, a second weaker peak larger by 16 Da, which very likely corresponded to the oxidized form of the same peptide, was also immunocaptured in the 3 patients’ sera (Fig. 4B). SELDI-TOF-MS spectra without immunocapture of the similar samples were illustrated for comparison (Fig. 4C).

7765 DA Peptide is PF4. In this study, we discovered a biomarker at 7765 Da that correlated with the impact of CT and had significant peak intensity decrease in post-CT sera (Fig. 2). Coincidently, a serum protein of the same molecular size, which was analyzed by SELDI-TOF MS with IMAC-Cu arrays, has previously been identified as platelet factor-4 (PF4) by tandem mass spectrometry and immunoaffinity capture assay by a coauthor of the present paper (12). Therefore, following the same procedure for immunoaffinity capture assay, we investigated whether the present 7765-Da CT-related biomarker could be PF4 in 2 pairs of NPC patients’ sera collected before and after CT (Fig. 5A). Anti-PF4 antibody specifically immunocaptured a protein peak at 7765 Da in the 2 pre-CT but not in the post-CT sera. This immunoassay reactivity pattern in NPC (Fig. 5A) strongly correlated with that observed in experiments using IMAC-Cu arrays without capture (Fig. 5B), confirming that the 7765 Da protein is indeed PF4.

Discussion

In this ProteinChip array profiling study, 13 serum protein/peptide biomarkers were correlated with different clinical parameters in NPC patients. Similar proteomics studies in head and neck cancer (HNC) patients were also previously reported (13–15). To compare the biomarkers...
commonly expressed in both NPC and HNC, we matched, using <0.3% mass difference tolerance level, the molecular masses of the 32 HNC-associated marker peaks reported in one paper (14) with the present 13 NPC-associated marker peaks and those in lower ranks of significance in our original database (data not shown). Two biomarkers (2951 and 7767 Da) in the HNC panel were overlapped with a CT response-associated peptide (2950 Da) (Fig. 3) and a biomarker (7765 Da) associated with the impact of CT (Fig. 2), which was identified to be PF4 (Fig. 5). Nine NPC-associated marker peaks (2785, 2950, 3776, 3889, 4180, 4467, 5062, 5081, and 7989 Da) in lower rank of significance also had molecular masses analogous with those in the HNC panel (2778, 2951, 3772, 3888, 4181, 4464, 5064, 5078, and 7971 Da), raising the interesting possibility of extending the clinical applications of these biomarkers to HNC if they turn out to be similar markers on protein identity study.

Two small serum peptides (2803 Da and 3953 Da) were significantly increased in NPC patients both at relapse...
and primary diagnosis. With either peptide alone, diagnostic sensitivities of 94% or 100% and specificities of 97% or 94%, respectively, were achieved. The sensitivities are higher than that achieved by the conventional serological test for NPC, the Epstein-Barr virus viral capsid antigen IgA antibody test (81%) (16) and are roughly equivalent to that of the PCR test for circulating plasma Epstein-Barr virus DNA (95%) (17). The specificities are similar to those reported in both tests (96% and 98% respectively). The consistent presence of these 2 serum peptides both at initial diagnosis and relapse is, however, distinguished from SAA, which was increased only at the time of relapse but not or at a much lower extent at initial diagnosis (6). Furthermore, unlike SAA, the 2 peptides persist at lower but significant concentrations in patients in remission. This profile is also very different from that of plasma Epstein-Barr virus DNA, which invariably drops to an undetectable level during remission after RT (18).

The peptide at 3953 Da was identified to be a cleaved fragment of the precursor of ITIH-4. Although this intertrypsin inhibitor was commonly found in disease conditions such as sepsis (19), it was also increased in hepatoma (20), lung cancer (21), brain tumor (22), and sera/plasma of ovarian and pancreatic cancer patients (23, 24). The fragmentation pattern was proposed to be useful in the classification of ovarian cancer (25), and it was suggested that cancer specificity in ITIH4 could perhaps be conferred by posttranslational modifications such as differential proteolytic truncation (26). Tumor-associated macrophages can induce chronic inflammation, which, in turn, can enhance cancer progression and metastases (27). This may explain the large quantity of inflammation-related molecules present in the sera of NPC patients at relapse. The fact that cleaved intertrypsin inhibitor fragments could inhibit granzyme K, which is a lymphocyte serine protease implicated in T cell- and natural killer cell-mediated cytotoxic defense reactions (28) suggested the possibility that this cleavage may perhaps inactivate cytotoxic defense, resulting in development of primary cancer and its relapses.

CT can kill tumor cells but also impair the immune system, leading to acute depletion of immune-mediating
and tumor-associated proteins after systemic treatment. Incidentally, 5 of the 7 biomarkers (7588, 7659, 7765, 7843, and 8372 Da) were significantly decreased after CT (Figs. 2). One of them (7765 Da) was subsequently identified as PF4, which is a CXCL4 chemokine expressed in various inflammation cell types (29) as well as metastatic prostate cancer cells (30) and megakaryoblastic leukemia cells (31). Concurring with our findings, plasma PF4 concentrations have been found to increase in megakaryoblastic leukemia (31) and other cancers (32) during active disease or relapse but return to reference values during remission after CT.

In this study, a reasonably satisfactory classification tree was achieved conferring 87% specificity and 80% sensitivity in CT-response prediction using the 2 biomarkers at 2950 Da and 6701 Da (Fig. 3C and Table 2). Despite the drawback of a lack of tumor specificity in some proteomics markers (33), we believe that this should not undermine its usefulness in CT response analysis. This notion can be shown by vigorous exploration of the clinical applications of many host targets of ubiquitous nature in known pathways of apoptosis, protein kinase, and NF-κB in CT response analysis. However, simply extrapolating the CT response relationship for the present biomarkers to predict that of other cytotoxic drugs, or to predict response in other cancers for the same drugs can be fraught with potential errors because the CT response relationship can be drug- or tumor-type specific or both. This notion can be exemplified by a marker, BRCA1, whose lack of protein expression was associated with chemosensitivity to DNA-damaging agents but chemoresistance to microtubule interfering agents (34), whereas the protein expression of p53 is correlated with chemoresistance in non-small cell but not small cell lung cancer (35).

Variability of the SELDI-TOF-MS technique was reported (36). A multinstitutional reproducibility evaluation in prostate cancer reported a range of CVs of 15%–36% in marker peak intensities (37) compared with CVs of 8%–26% in our study (9). Our higher consistency is probably attributed to the use of a single well-calibrated SELDI-TOF-MS platform instead of the multiple platforms used in the previous report. Recently, a number of studies on the influence of preanalytical variables in proteomics techniques (38, 39) were reported. We anticipate that more international efforts in quality control will gradually set a standard to further improve the consistency of these proteomics techniques, thus enabling the use of new protein biomarkers to complement various conventional tests in predicting CT response.

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