Identification of Serum Amyloid A Protein As a Potentially Useful Biomarker to Monitor Relapse of Nasopharyngeal Cancer by Serum Proteomic Profiling


Abstract

Purpose: Nasopharyngeal cancer (NPC) is a common cancer in Hong Kong, and relapse can occur frequently. Using protein chip profiling analysis, we aimed to identify serum biomarkers that were useful in the diagnosis of relapse in NPC.

Experimental Design: Profiling analysis was performed on 704 sera collected from 42 NPC patients, 39 lung cancer patients, 30 patients with the benign metabolic disorder thyrotoxicosis (TX), and 35 normal individuals (NM). Protein profile in each NPC patient during clinical follow up was correlated with the relapse status.

Results: Profiling analysis identified two biomarkers with molecular masses of 11.6 and 11.8 kDa, which were significantly elevated in 22 of 31 (71%) and 21 of 31 (68%) NPC patients, respectively, at the time of relapse (RP) as compared with 11 patients in complete remission (CR; RP versus CR, P = 0.009). The markers were also elevated in 16 of 39 (41%) lung cancer patients at initial diagnosis. By tryptic digestion, followed by tandem mass spectrometry fragmentation, the markers were identified as two isoforms of serum amyloid A (SAA) protein. Monitoring the patients longitudinally for SAA level both by protein chip and immunoassay showed a dramatic SAA increase, which correlated with relapse and a drastic fall correlated with response to salvage chemotherapy. Serum SAA findings were compared with those of serum Epstein-Barr virus DNA in three relapsed patients showing a similar correlation with relapse and chemo-response.

Conclusions: SAA could be a useful biomarker to monitor relapse of NPC.

Introduction

Nasopharyngeal cancer (NPC) is the fourth-most prevalent cancer among the male population in Hong Kong (1). Although NPC is very responsive to radiation therapy (RT; see Ref. 2), relapse frequently occurs and is one of the main concerns in the treatment of NPC. For instance, a retrospective analysis performed in our hospital involving >5000 NPC patients showed that, although RT could achieve complete response in 83% of the patients, resulting in remission of disease lasting >6 months, 53% of these patients later developed either loco-regional or distant relapse at a median period of 1.4 years posttreatment (3). For the patients who developed relapses to distant organs such as liver, lung, or bone, the outcome was generally ominous, with >98% of the patients eventually dying of disease (4). Obviously, there is an urgent need to find markers that can diagnose relapse early, allowing salvage treatment to be implemented hopefully in time to treat the malignancy when tumor load is still small.

Making use of a comprehensive serum bank collected longitudinally from a cohort of NPC patients with relapse, we have reported previously that we could detect relapse by amplification of serum circulating Epstein-Barr virus (EBV) DNA using real-time quantitative PCR (Q-PCR; see Ref. 5). By this Q-PCR technique, the serum EBV DNA was also found to be a potential surrogate marker for monitoring a subgroup of EBV-positive lung cancer, lymphoepithelioma-like lung carcinoma (6). In this report, we analyze the serum protein components from NPC patients by surface-enhanced laser desorption and ionization-time of flight-mass spectrometry or, in short, protein chip profiling (7). Instead of studying one protein at a time by the conventional approach, this profiling technology coupled with various powerful computer biomarker classification algorithms enabled us to rapidly analyze tens of thousands of complex proteins from 704 sera in this study. A report jointly published in the United States by the National Cancer Institute and the Food and Drug Administration has demonstrated the remarkable potential of this proteomic technology in the diagnosis of ovarian cancer (8). In the present report, we further expand the application of this novel technology by identifying protein markers, which were strongly correlated with the events of relapse using a cohort of NPC patients with comprehensive clinical follow up.
Materials and Methods

**Patients, Treatment Protocols, and Serum Samples.** Thirty-one NPC patients who had recurrent undifferentiated carcinoma of the nasopharyngeal type or poorly differentiated squamous cell carcinoma were entered into a prospective Phase II clinical study using gemcitabine and cisplatin combination chemotherapy. These patients previously received primary external megavoltage RT with curative intent after initial diagnosis of NPC, but suffered from either loco-regional recurrences or distant metastases post RT. Briefly, the RT treatment protocol used (9) was as follows. Well-collimated photon beams from a 4–6 MV linear accelerator were used to encompass the nasopharynx and its adjacent regions, such as the posterior nasal fossae, parts of the paranasal sinuses, parapharyngeal spaces, and the skull base. A custom-made shell of Cobex or thermoplastic material was used for beam direction and immobilization. The initial phase of the radiation was delivered through two laterally opposing en bloc facio-cervical fields covering the nasopharyngeal region and the upper-cervical lymphatic regions, whereas the lower-cervical lymphatic regions were irradiated through a single anterior cervical field with a central median shield to spare the spinal cord. This was followed by the second phase, in which small split-facial fields were used to spare the lateral portion of the temporal lobes of the brain and the temporal-mandibular joints. The lower-anterior cervical field was extended superiorly to include the entire neck. The total dose to the target volume was 66 Gy to the nasopharynx and ~60–66 Gy to the neck. When necessary, a single posterior-lateral field was used to boost the parapharyngeal space where slight under-dosing by the small split-facial fields used in the second phase might have taken place.

The relapsed patients were treated on an outpatient basis by a chemotherapy protocol approved by the Queen Elizabeth Hospital Ethics Committee in accordance with the Helsinki Declaration. Informed written consent was obtained from all patients before enrollment (10). Gemcitabine was administered at a dose of 1000 mg/m² by i.v. infusion in 250 ml of normal saline over 30 min on days 1, 8, and 15 of a 28-day cycle, preceded by bolus i.v. injection of 20 mg of metoclopramide and 5 mg of dexamethasone. Cisplatin, at a dose of 50 mg/m², was given i.v. in 1000 ml of normal saline over 2 h after gemcitabine infusion on days 1 and 8, again preceded by bolus i.v. injection of 20 mg of metoclopramide, 5 mg of dexamethasone, and 20 mg of frusemide. Patients received at least three cycles of chemotherapy before being formally assessed for response. In responding patients, treatment could be continued for more than six cycles until there was no further clinical benefit from chemotherapy. However, treatment could be discontinued for patients with further disease progression, unacceptable drug toxicity, or after achievement of the best response.

Serum samples were collected from the relapsed patients at the initial diagnosis of disease during and after the course of treatment prospectively for this protein chip profiling study. However, only 20 of 31 relapsed patients had sera available at diagnosis. Serum collection was started in the remaining 11 patients only at relapse. The patients were followed up for a period of 2–6 years, and a total of 425 blood samples were longitudinally collected during this follow-up period. Another 11 NPC patients, who were treated by a similar RT protocol and were monitored for at least 8 years, were also entered into this study. These patients did not develop any relapse and were in complete remission of disease during the entire follow-up period. No chemotherapy was administered in this group. These individuals served as the complete remission group. Two hundred ten blood samples were collected longitudinally from this group during the monitoring period. Thirty-nine lung cancer patients with sera available from our serum bank were randomly included to serve as other cancer controls. Detailed clinical manifestations were not, however, available in these patients.

Thirty patients with the benign metabolic disorder thyrotoxicosis (TX), and 35 normal individuals served as noncancer controls. One hundred four single-point serum samples (39 from lung cancer patients, 30 from TX patients, and 35 from normal controls) were obtained from these three groups. Eight milliliters of blood were collected with patient consent from each sample for this molecular study. Briefly, each blood sample was allowed to clot and was centrifuged at 1500 × g for 10 min. Sera were collected, aliquoted, and kept frozen at ~ 70°C until the analysis was carried out. This molecular study was also approved by the Queen Elizabeth Hospital Ethics Committee.

**Protein Chip Profiling Analysis.** Serum samples were thawed, and 20 μl of each serum were denatured by adding 30 μl of 50 mM Tris-HCl buffer containing 9 M urea and 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (pH 9). The proteins were fractionated in an anion-exchange Q HyperD F 96-well filter plate (Ciphergen Biosystems, Fremont, CA). Six fractions (namely fractions from the flow through pH 7, pH 5, pH 4, and pH 3 and organic eluant fractions) were collected by stepwise decrease in pH. The fractions were diluted and profiled on a Cu (II) Immobilized Metal Affinity Capture (IMAC3) Protein Chip Array (Ciphergen Biosystems, Fremont, CA; see Ref. 11). All fractionation and profiling steps were performed on a Biomek 2000 Robotic Station (Beckman Coulter). Sinapinic acid (Ciphergen Biosystems, Fremont, CA), which served as energy absorbing molecule, was used for facilitating desorption and ionization of proteins on the protein chip array. Data acquisition was performed by a Protein Biology System, which included a ProteinChip Reader (Model PBS; II; Ciphergen Biosystems, Fremont, CA). A protein repertoire map was generated in which the individual proteins were displayed as unique peaks based on their mass and charge (m/z). Serum samples from NPC and lung cancer patients and the noncancer controls were run concurrently to minimize experimental variations.

**Tryptic Digestion and Protein Identification.** Proteins fractionated at organic solvent eluant from the anion-exchange filters were further characterized by SDS-PAGE (12). The bands at approximately 11.6 and 11.8 kDa containing the desired biomarkers were cut from the gel and subjected to overnight tryptic digestion in a reaction volume of 20 μl of 50 mM ammonium bicarbonate buffer containing 1 pmol of trypsin at pH 8.0 (13). Subsequently, 2 μl of the digestion mixture were analyzed on a Normal Phase ProteinChip Array (NP2). α-Cyano-4-hydroxycinnamic acid (Ciphergen Biosystems, Fremont, CA) was used to facilitate desorption/ionization of the peptides generated from the tryptic digest. Masses of the tryptic-digested peptides were measured by the ProteinChip Reader and
were used for initial protein identification from the Profound database. The identities of the proteins were further confirmed by tandem mass spectrometry (MS/MS) fragmentation analysis of a peptide at 2177.9 Da generated from the tryptic digest on an ABI Q-Star quadrupole tandem mass spectrometer equipped with a Ciphergen ProteinChip Interface (PCI-1000) as described previously (14). MS/MS fragmentation of this peptide generated a set of MS/MS ion fingerprint data that was then entered into a mass spectrometry database search engine, Mascot, to find the closest match with known proteins in this database as reported previously (15).

**Immunooassay of SAA.** After confirming that the proteins of interest were the two isoforms of SAA, an immunooassay kit (Biosource, Belgium) was used to quantify the level of SAA. The assay was performed according to the manufacturer’s instructions. Briefly, 200 μl of sera in duplicates at a dilution range of 1:1000 to 1:40,000, together with SAA standards at concentrations of 4.7–300 ng/ml, were coated onto the immunooassay plates. After washing, an alkaline phosphatase conjugated anti-SAA antibody, which is reactive to all isoforms of human SAA, was added, and the reaction mixture was incubated for 1 h at 37°C. After washing, the substrate for alkaline phosphatase, p-nitro-phenyl phosphate, was added, and the colorimetric reaction was terminated after incubation for 1 h at 37°C. The immunooassay plate was read at 405 nm in a Bio-Rad 3550 microplate reader. The concentration of SAA in the sera from patients was determined from the SAA standard curves.

**Q-PCR of Serum EBV DNA.** Q-PCR of serum circulating EBV DNA was performed in three NPC patients in relapse and five patients in remission by a method as described previously (5). Briefly, DNA was first extracted from 200–400 μl of each serum from the longitudinally monitored patients by a QIAamp blood DNA isolation kit (Qiagene, Hilden, Germany). Q-PCR for the extracted DNA was performed by the TaqMan technique with a pair of primers specific for DNA encoding EBV small RNA (EBER-1) using DNA from an EBV-positive Burkitt’s lymphoma cell line, Nalmalwa (containing two genome copies of EBV per cell), as a positive control and an EBV-negative Burkitt’s lymphoma cell line, BJAB, as a negative control. The results were compared with those of SAA.

**Bioinformatics and Statistical Methods.** The protein chip profiling spectra from the serum samples were collected and analyzed by Ciphergen ProteinChip Software 3.0.2 (16). Using the Biomarker Wizard Mode, consistent biomarker peak sets across multiple serum samples after normalization were compared with searches for biomarkers that were differentially elevated in the relapse group relative to the remission group or vice versa according to the instruction manual of the software. A nonparametric Mann-Whitney U test (17) was used to compare the peak intensities of the protein profiling results from the different groups.

**Results**

**Protein Chip Array Profiling Analysis.** Each serum sample from NPC patients was first separated into six fractions by anion-exchange filter fractionation, and >1000 biomarkers were resolved from each serum by protein chip profiling. After analyzing the serum protein profiles, we identified two biomarkers at approximately 11.6 and 11.8 kDa from the organic solvent eluant fraction, which were abundantly present in a patient at relapse (Fig. 1A, top plot), but not in another patient who was in complete remission (Fig. 1A, bottom plot). After we tested more patients, we observed an elevation of these two markers in the majority of patients at relapse (Fig. 1B, left plot) but not in remission (Fig. 1B, right plot). In contrast, the levels of two other biomarkers at ∼14 kDa were equally increased in patients at both relapse and remission (Fig. 1B). To summarize the profiling findings in Fig. 2, the average peak intensities (mean ± SD) of the 11.6- and 11.8-kDa biomarkers in 31 NPC

---

3 Internet address: prow1.rockefeller.edu.
4 Internet address: www.matrixscience.com/home.html.
patients at relapse (21 ± 25 and 22.5 ± 27.2, respectively) were significantly higher than those in 11 NPC patients during complete remission (1.6 ± 1.1 and 1.7 ± 1.0, respectively; \( P = 0.009 \) for both biomarkers by Mann-Whitney U test), 30 patients with the benign metabolic disorder TX, (1 ± 1 and 1.4 ± 1.4, respectively; \( P < 0.001 \)), or 35 normal individuals (0.2 ± 0.2 and 0.3 ± 0.1, respectively; \( P < 0.001 \)). Only 20 of 31 relapsed patients had sera available at the initial diagnosis of NPC. The mean intensities of the 11.6- and 11.8-kDa biomarkers measured at diagnosis in these relapsed patients (0.8 ± 1.1 and 1.2 ± 2.5, respectively) were not significantly elevated as compared with the remission group at diagnosis (0.5 ± 0.4 and 0.7 ± 0.7, respectively; \( P > 0.05 \)) or the TX controls (1 ± 1 and 1.4 ± 1.4, respectively; \( P > 0.05 \)), although the biomarkers were significantly increased over the normal controls by small differences (0.8 and 1.2 versus 0.2 and 0.3; \( P \leq 0.001 \)). This illustrated that the identified biomarkers were largely up-regulated at relapse.

### Identities of the Biomarkers

To determine the identities of the two biomarkers, we first resolved serum proteins by SDS-PAGE. Two protein bands of interest at 11.6 kDa and 11.8 kDa were cut from the gel for subsequent tryptic digestion. The peptide map after tryptic digestion is illustrated in Fig. 3.A. MS/MS fragmentation analysis of a peptide at 2177.9 Da generated from the tryptic digest (Fig. 3A, boxed peak) was further carried out on a tandem mass spectrometer as described in “Materials and Methods” to find the identities of the biomarkers (14). A MS/MS ion fingerprint was generated (Fig. 3B) and entered into a mass spectrometry Internet search engine database, Mascot, to find the closest match to its ion pattern with existing proteins in the database (15). Thirty-four of 37 ions in the fingerprint generated from each of the two proteins matched with 100% probability (i.e., 1.0 e\(^{-4}\) 000; Fig. 3B, inset table) with human SAA illustrating that they were, in fact, two different isoforms of SAA.

### Longitudinal Serum Follow Up by Protein Chip, Immunoassay, and Q-PCR

With the two SAA isoforms being predominantly elevated at relapse in NPC patients, an immunoassay for SAA was also developed to test the ability of the assay to monitor relapse in parallel with protein chip profiling by longitudinal serum follow up in more patients. Circulating serum EBV DNA, which was previously shown by us (5) and an other group (19) to be useful in the diagnosis of relapse of NPC, was also tested using Q-PCR. Biomarker serum monitoring by the three techniques in three relapsed patients is shown in Fig. 4, A–C, whereas 11, 5, and 8 patients in remission were monitored by protein chip, immunoassay and Q-PCR respectively, as shown in Fig. 4D. In the first relapsed patient under serological monitoring, EBV DNA was elevated at diagnosis, but not at relapse. In the other two patients, EBV DNA was also not elevated at relapse. The protein chip and immunoassay results in these patients were also consistent with these findings (Fig. 4, A–C).
follow-up (Fig. 4A), the two SAA peaks (11.6 and 11.8 kDa) by protein chip were significantly elevated at diagnosis of NPC. The two peaks were subsequently increased at clinical diagnosis of distant metastases to bone, liver, and spleen (Fig. 4A). After salvage chemotherapy with gemcitabine and cisplatin and radiotherapy to bone metastasis, the tissue metastases were decreased to background levels. The metastases were elevated after further progression of the disease in liver, shortly before the death of the patient. After serum monitoring by immunoassay for SAA and Q-PCR for serum EBV DNA, a similar rising pattern with relapse/tumor progression and a falling trend with chemotherapy was also found (Fig. 4A).

In the second patient, sera were obtained only after distant metastases in liver and distant lymph nodes were diagnosed (Fig. 4B). The two SAA peaks by protein chip were already substantially elevated at metastases. The protein levels again subsided after chemotherapy, but were increased and then leveled off at a significantly higher level after documentation of only a partial response to the treatment in liver and a complete response in the distant lymph nodes (Fig. 4B). After further disease progression in the two distant sites before the patient died, the two peaks were elevated to a higher level. Similar trends of correlation with clinical relapse and response to salvage chemotherapy were also observed in SAA immunoassay and EBV DNA Q-PCR. The difference between EBV DNA and SAA monitoring in this patient was that a substantially higher peak of serum EBV DNA was detected at the initial relapse, but the level of SAA rose earlier than EBV DNA postchemotherapy on progression.

In the third NPC patient, low and almost background levels of SAA and EBV DNA were both detected at initial diagnosis of NPC (Fig. 4C). On bone metastasis and further progression, the

![Table 1](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex/Age (yr)</th>
<th>Stages&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sites of recurrence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Chemoresponse&lt;sup&gt;c&lt;/sup&gt;</th>
<th>At diagnosis</th>
<th>At Rec/Prog</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall</td>
<td>TNM&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>11.6 kDa</td>
<td>11.8 kDa</td>
</tr>
<tr>
<td>1</td>
<td>M/56</td>
<td>4 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Bilat Ax LN Met</td>
<td>PR</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>M/60</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>NP and BOS Rec</td>
<td>PR</td>
<td>5.2</td>
<td>11.6</td>
</tr>
<tr>
<td>3</td>
<td>M/31</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Bone, Liver, and Spleen Met</td>
<td>PR</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>M/37</td>
<td>4 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>NP and Neck LN Rec; Liver, Abd LN, Lung and Bone Met</td>
<td>PR</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>5</td>
<td>M/56</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lung and Med LN Met</td>
<td>CR</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>M/47</td>
<td>4 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Liver, Plee, and Grom LN Met</td>
<td>SD</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>7</td>
<td>M/47</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>NP and Neck LN Rec</td>
<td>SD</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>8</td>
<td>F/60</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Ax and Inflav LN Met</td>
<td>CR</td>
<td>1.3</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>M/51</td>
<td>4 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Neck LN Rec; Liver, Lung, and Abd LN Met</td>
<td>PR</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>10</td>
<td>M/55</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Liver and Abd LN Met</td>
<td>PR</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>11</td>
<td>M/45</td>
<td>2 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>NP Rec, Lung Met</td>
<td>CR</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>12</td>
<td>M/45</td>
<td>4 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Bone, Lung and Liver Met</td>
<td>NA</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>13</td>
<td>M/49</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lung Met</td>
<td>PR</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>14</td>
<td>M/40</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>NP and BOS Rec</td>
<td>PR</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>15</td>
<td>M/51</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Bone Met</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>16</td>
<td>M/41</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>NP and BOS Rec</td>
<td>SD</td>
<td>0.8</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
<td>M/61</td>
<td>2 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Abd LN Met</td>
<td>PR</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>18</td>
<td>M/63</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>NP and BOS Rec</td>
<td>PR</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>19</td>
<td>M/50</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lung and Pleur Met</td>
<td>PR</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>20</td>
<td>M/43</td>
<td>5 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>NP and BOS Rec; Lung and Pleur Met</td>
<td>PR</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>21</td>
<td>M/46</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lung Met</td>
<td>SD</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>22</td>
<td>M/51</td>
<td>2 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>NP and BOS Rec</td>
<td>CR</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>23</td>
<td>M/38</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lung and Med LN Met</td>
<td>CR</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>24</td>
<td>F/47</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lung Met</td>
<td>PR</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>25</td>
<td>M/42</td>
<td>2 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lung Met</td>
<td>CR</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>26</td>
<td>M/64</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lung, Liver and Bone Met</td>
<td>PR</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>27</td>
<td>M/59</td>
<td>4 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Neck LN Rec; Liver, Lung, Bone, Med and Abd LN Met</td>
<td>PR</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>28</td>
<td>F/34</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>NP and BOS Rec; Lung, Bone and Abd LN Met</td>
<td>PR</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>29</td>
<td>M/34</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>NP and BOS Rec</td>
<td>SD</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>30</td>
<td>M/50</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lung and Pleur Met</td>
<td>PR</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>31</td>
<td>M/38</td>
<td>3 T&lt;sub&gt;N&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lung Met</td>
<td>PR</td>
<td>0.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Staging classification according to the staging system of Ho (18).
<sup>b</sup> Sites of recurrence: Bilat Ax LN Met, bilateral axillary lymph node metastasis; NP and BOS Rec, nasopharyngeal and base of skull recurrence; Abd LN Met, abdominal lymph node metastasis; Med LN Met, mediastinal lymph node metastasis; Pleur Met, pleural metastasis; Infclav LN Met, infraclavicular lymph node metastasis.
<sup>c</sup> Chemo-response: CR, complete response; PR, partial response; SD, static disease; NA, not available.
<sup>d</sup> TNM, Tumor-Node-Metastasis; Rec/Prog, recurrence or progression of disease.
levels of the two markers were not significantly elevated, although the time when the sera were collected was actually intermingled with the RT treatment (Fig. 4C). More than 2 months before the spread of the disease to groin lymph nodes, other distant lymph nodes and liver (Fig. 4C), both SAA and EBV DNA were elevated in a similar fashion. After chemotherapy, the level of the two markers were again decreased down to background levels, as in the previous two patients. A slight increase of SAA was noted before further disease progression to liver and bone. However, the patients soon died of disease, without providing additional sera samples for testing.

Longitudinally monitoring 11 patients in complete remission for >2500 days by protein chip and five and eight patients in remission, respectively, by SAA immunoassay and EBV DNA Q-PCR showed no substantial elevation of the two markers as in relapsed patients (Fig. 4D), thus providing further evidence that serum SAA elevation was relapse associated.

Discussion
Modern mass spectrometry has revolutionized the study of proteomics by enabling rapid and automatic resolution of thousands of biomarkers from many patients in a short period of time. Surface-enhanced laser desorption and ionization-time of flight-mass spectrometry or protein chip profiling has further advanced the technology greatly by combining mass spectrometry with a surface-enhanced biochip, which allows uniform and reproducible binding and desorption of biomarkers, and, therefore, quantitative comparison of individually resolved biomarkers in different cancers can be performed (7, 20–22). This protein chip profiling technique was adopted to detect biomarkers in prostate cancer (23, 24), head and neck cancer (25), lung cancer (26), breast cancer (27, 28), and pancreatic cancer (29). Coupled with different powerful computer algorithms, the biomarkers identified can be rapidly correlated with various clinical and pathological parameters of the cancer patients, thus allowing them to be applied rapidly in diagnostic or therapeutic use. For instance, through neural-network training on a complex pattern of multiple proteins, a proteomic-classification algorithm, which could sensitively and specifically identify even early stages of ovarian cancer, was discovered (8, 30).

Relapse of disease remains one of the biggest problems in clinical management of cancer. In NPC, more than half of the patients with complete response to RT developed relapse after further follow up. By the time distant relapse is diagnosed clinically, it is usually hard to manage. Therefore, finding biomarkers to diagnose relapse early is an important undertaking in translational cancer research. Nevertheless, studies reporting the use of protein chip profiling or other mass spectrometry techniques for discovering cancer diagnostic markers frequently give little emphasis on the disease-monitoring role of these biomarkers. With our comprehensive serum bank that was collected longitudinally from NPC patients, we were able to explore the monitoring role of the biomarkers by the proteomic approach of protein chip profiling. The levels of biomarkers determined at different time points of the clinical course of the patients can reveal important information on the potential clinical usefulness of the biomarkers.

In the present study, two biomarkers were identified by protein chip profiling to be strongly correlated with the clinical relapse status in NPC patients. Subsequent peptide mapping and MS/MS fragmentation identified the biomarkers as two isoforms of SAA, whereas SAA immunoassay confirmed the protein chip results. SAA is an acute-phase protein existing as either pseudogenes or those with no transcript or protein expression. In normal individuals, SAA is produced by hepatocytes in the liver (33). After its production, it is secreted into serum and rapidly binds to high-density lipoprotein, with 90% of the protein particles being high-density lipoprotein bound (34). A review of the literature showed that only a low level of SAA can be found in the sera of healthy normal individuals, despite the ubiquitous nature of SAA (35). This is in sharp contrast to the patients with neoplastic diseases, such as those with renal (36) and colorectal (37) cancer and many others, who showed dra-

---

Table 2  Clinical parameters and peak intensities of the 11.6- and 11.8-kDa biomarkers in the remission group of nasopharyngeal cancer patients under study

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex/Age (yr)</th>
<th>Overall</th>
<th>TNM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Follow-up period (mo)</th>
<th>Peak intensities of two biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>At diagnosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.6 kDA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>96</td>
<td>1</td>
</tr>
<tr>
<td>32</td>
<td>F/35</td>
<td>2</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>100</td>
<td>0.8</td>
</tr>
<tr>
<td>33</td>
<td>M/59</td>
<td>3</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;aN&lt;sub&gt;2&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>102</td>
<td>0.04</td>
</tr>
<tr>
<td>34</td>
<td>F/51</td>
<td>2</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>114</td>
<td>0.3</td>
</tr>
<tr>
<td>35</td>
<td>M/40</td>
<td>3</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>104</td>
<td>0.8</td>
</tr>
<tr>
<td>36</td>
<td>M/44</td>
<td>3</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>97</td>
<td>1.3</td>
</tr>
<tr>
<td>37</td>
<td>M/42</td>
<td>2</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>105</td>
<td>0.2</td>
</tr>
<tr>
<td>38</td>
<td>M/52</td>
<td>2</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>112</td>
<td>0.4</td>
</tr>
<tr>
<td>39</td>
<td>M/44</td>
<td>3</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;aN&lt;sub&gt;2&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>113</td>
<td>0.1</td>
</tr>
<tr>
<td>40</td>
<td>F/43</td>
<td>3</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>98</td>
<td>0.5</td>
</tr>
<tr>
<td>41</td>
<td>F/45</td>
<td>3</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>105</td>
<td>0.4</td>
</tr>
<tr>
<td>42</td>
<td>M/48</td>
<td>2</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>105</td>
<td>1.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Staging classification according to the staging system of Ho (18).
<sup>b</sup> TNM, Tumor-Node-Metastasis.

---

48  Proteomic Analysis Identifies SAA as a Marker for NPC Relapse
matic elevation of serum SAA. In line with the findings of this study, prognostic significance of SAA in other cancers were also found by conventional radioimmunoassay (38). For instance, it was reported that there was a 500-fold increase in SAA levels during active disease status in prostate cancer patients when compared with those in remission (39). This was much more sensitive than another prostate cancer marker, acid phosphatase, which was only elevated 10-fold. Furthermore, similar to the present findings, serum SAA level by two-point analysis was also found to be significantly higher in patients with metastatic disease than those with limited disease among 10 classes of solid tumors and three classes of hematological malignancies using solid-phase competitive radioimmunoassay (40). Instead of two-point assessment, our present study demonstrated, for the first time, with comprehensive longitudinal follow up, that a gradual elevation of SAA occurred on distant metastasis of NPC to bone, lung, or liver and that a dramatic decrease of the protein to background level on salvage chemotherapy occurred. This biomarker was only present in low levels in NPC patients with >8 years of remission, thus demonstrating it to be a potential candidate for monitoring relapse.

SAA is not considered as a cancer-specific marker per se. Apart from disseminated malignancy, its elevation was also reported as part of the body’s response to various injuries or other diseases, including trauma, infection, inflammation, rheumatoid arthritis, and amyloidosis (41). However, in the 31 relapsed NPC patients studied in this project, infection resulting in neutropenic fever was found to be associated with SAA elevation on only two occasions. Therefore, the majority of the SAA elevation in the relapsed patients appeared to be unrelated to the benign cause of infection and inflammation, but was relapse associated. Our results are consistent with the other studies, one of which reported that conventional radioimmunoassay in 621 cancer patients free of inflammation also showed a substantial increase of SAA level in 95% (281 of 289) of patients with metastatic solid tumors, in all myelocytic leukemia patients with high leukocyte counts, and in all advanced lymphoma patients (42).

The reasons for the association of an acute-phase protein with cancer relapse remain largely unknown. We did not observe any protein expression by in situ immunostaining using an anti-SAA polyclonal antibody in a biopsy section from one NPC patient, although substantial staining was observed in a control liver resection from a noncancer patient. This suggested that serum SAA level might not be a primary product produced by the tumor lesions, but could be a secondary product produced by the hepatocytes. It was reported previously that injection of bacterial DNA into mice can rapidly induce SAA production.
Fig. 4 Longitudinal monitoring of serum amyloid A (SAA) protein level by protein chip profiling and immunoassay and circulating serum Epstein-Barr virus (EBV) DNA by real-time quantitative PCR (Q-PCR) in nasopharyngeal carcinoma (NPC) patients. A, B, and C, three NPC patients in relapse were monitored by SAA protein chip (SAA Protein Chip), SAA enzyme immunoassay (SAA EIA), and EBV DNA by Q-PCR (EBV DNA Q-PCR). D, 11, 5, and 8 patients in remission were also monitored by the three techniques, respectively. ▲ and △, follow-up profiling curves of 11.6 and 11.8 kDa SAA isoforms by protein chip; ▼ and ○, follow-up curves of SAA protein by immunoassay; ■ (EBER1), follow-up of serum EBV DNA encoding EBV small RNA-1 by Q-PCR; ■ BN, bone metastasis; ■ CR, complete response to chemotherapy; ■ CT, salvage chemotherapy; ■ DLN, distant lymph node metastasis; ■ DX, histopathological diagnosis of NPC; ■ Groin LN, metastasis in lymph nodes of the groin; ■ LV, liver metastasis; ■ LV PR, partial response to chemotherapy in tumor lesion in metastatic liver; ■ DLN CR, complete response to chemotherapy in tumor lesion in metastatic distant lymph node; ■ PG, progression of disease; ■ PR, partial response; ■ RT, radiation therapy; ■ SAA, immunoassay curves for SAA protein; ■ SP, spleen metastasis.
Our group and others also showed that NPC patients at relapse had abundant circulating EBV DNA in sera (5 and 19, respectively). Therefore, one possible explanation for the elevated serum SAA in NPC patients in relapse (particularly in distant metastasis) would be that during systemic spread, EBV DNA from tumor cells can trigger the production of large amounts of SAA from the hepatocytes. Once produced and secreted into the blood stream, binding of SAA onto high-density lipoprotein can result in its stability and, thus, its elevated level in serum. This hypothesis is supported by a closely related pattern of change in magnitude and temporal sequence for both SAA and EBV DNA in the three relapsed NPC patients studied (see Fig. 4, A–C). We are now performing Q-PCR in more patients to delineate comprehensively the relationship between the two markers. A number of NPC tumor xenograft lines carrying EBV genomes were established in nude mice in our laboratory (44). It would be interesting to further investigate viral association of acute-phase proteins by manipulating the EBV expression in these NPC xenograft models.

Although an association between SAA elevation and relapse did exist, this may not be simply attributable to a large tumor load. This is because NPC patients with a spread of bulky tumor cells to regional cervical lymph nodes at first presentation of disease did not appear to have very high serum SAA when compared with those with systemic spread to lung, liver, or bone. A possible explanation could be the requirement of the systemic presence of the viral DNA in the circulation to trigger SAA production and secretion. It is interesting to note that 41% of the lung cancer control patients at presentation had SAA elevation. Although detailed clinical manifestations were not available in these lung cancer patients, the possibility of elevated biomarker level correlated with relapse or more progressive disease in this malignancy should be further analyzed in the future.

Despite its tremendous increase at relapse in NPC patients, SAA at first presentation of disease was not substantially elevated. Therefore, it did not appear to be a good biomarker for initial diagnosis of NPC. However, further analysis from thousands of biomarkers other than SAA in the six ion-exchange fractions showed that a cluster of biomarkers, when used in combination, can achieve a diagnostic specificity and sensitivity of >90%. A manuscript is under preparation to report this finding. Apart from SAA and serum EBV DNA, a number of other prognostic indicators for NPC, for instance, serum IgG antibody against the EBV ZEBRA protein (45) and DNA flow cytometry that determined tumor proliferative fractions (46) were also identified previously in our institute. By combining protein chip identified biomarkers with these existing indicators, it is possible to establish a multiple-marker prognostic index for monitoring NPC in the future. By diagnosing distant relapse early, it may be possible for us to implement salvage chemotherapy at an earlier phase. This could be more effective when the metastatic tumor load is still small at this stage.

Acknowledgments

We thank our Hospital Chief Executive, Dr. Lawrence Lai, for his continuous support in this project.

References


Unpublished data.

Manuscript in preparation.
Pretolomic Analysis Identifies SAA As a Marker for NPC Relapse


Identification of Serum Amyloid A Protein As a Potentially Useful Biomarker to Monitor Relapse of Nasopharyngeal Cancer by Serum Proteomic Profiling


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/1/43

Cited articles
This article cites 44 articles, 9 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/1/43.full#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/10/1/43.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.