

Featured Article

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

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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, $P = 0.009$), 30 TX (RP versus TX, $P < 0.001$), or 35 NM (RP versus NM, $P < 0.001$). 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.

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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 sinus, 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 posterolateral 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 tentate 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.³ 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,⁴ to find the closest match with known proteins in this database as reported previously (15).

Immunoassay of SAA. After confirming that the proteins of interest were the two isoforms of SAA, an immunoassay kit (Biosource, Belgium) was used to quantify the level of SAA in three NPC patients in relapse and five patients in remission. The assay was performed according to the manufacturer's instructions. Briefly, 200 μ l of sera in duplicates at a dilution range of 1:1,000 to 1:40,000, together with SAA standards at concentrations of 4.7–300 ng/ml, were coated onto the immunoassay 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 immunoassay 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 eight patients in remission by a method as described previously (5). Briefly, DNA was first extracted from 200–400 μ l of each serum from the longitudinally monitored patients by a QIAamp blood DNA isolation kit (Qiagen, 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 \sim 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 \pm SD) of the 11.6- and 11.8-kDa biomarkers in 31 NPC

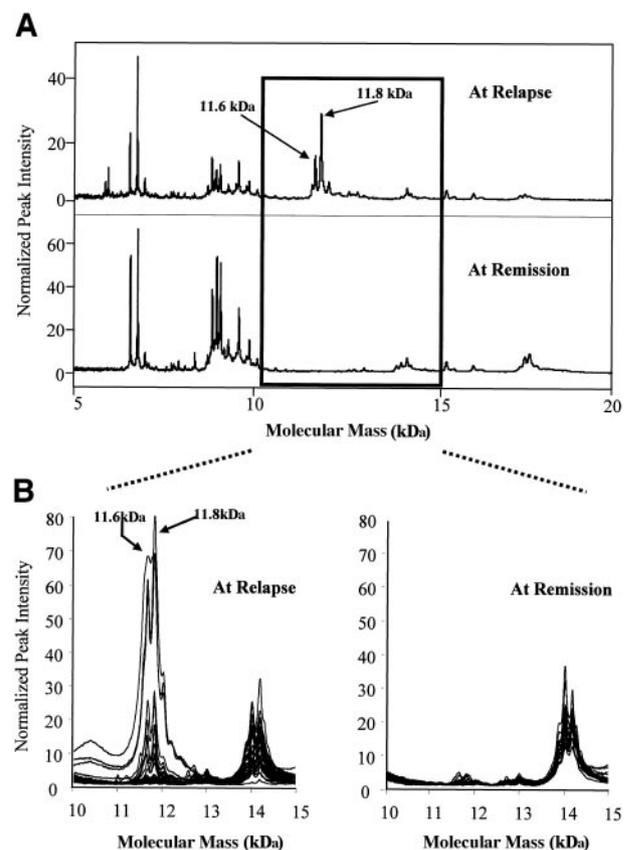


Fig. 1 Identification of serum biomarkers associated with relapse of nasopharyngeal carcinoma. **A**, two serum biomarkers found by protein chip profiling in a patient at relapse, but not in another patient who was in remission. **B**, overlapping of the two relapse-associated biomarker peaks in all patients studied. The two relapse-associated biomarkers identified were at a molecular mass of 11.6 and 11.8 kDa, whereas two control biomarkers with similar elevations in the two groups of patients were at \sim 14 kDa. More than 200 biomarkers were resolved from the organic solvent fraction, but only a small part of the protein retentate map at a molecular mass range of 5–20 kDa and 10–15 kDa is illustrated in **A** and **B**, respectively.

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⁴ Internet address: www.matrixscience.com/home.html.

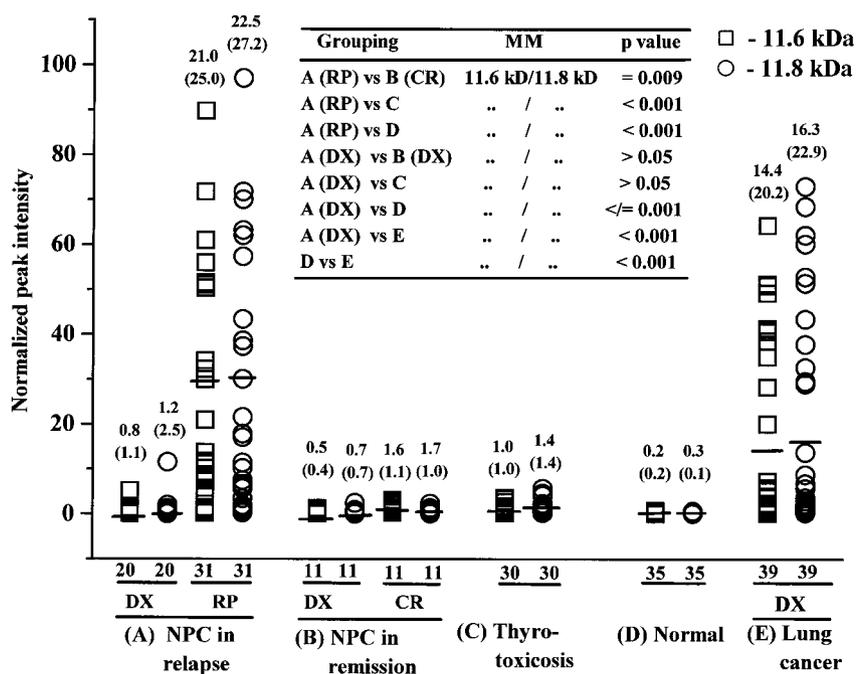


Fig. 2 Distribution of the peak intensities of the two protein-chip-identified biomarkers (11.6 and 11.8 kDa) in nasopharyngeal carcinoma (NPC) patients, lung cancer patients, patients with benign metabolic disease (thyrotoxicosis), and normal individuals. CR, biomarkers analyzed at complete remission of NPC; DX, biomarkers analyzed at initial diagnosis of NPC; RP, biomarkers analyzed at relapse of NPC. Mean peak intensities (\pm SD) are tabulated at the top of each column. Statistical comparisons of the mean peak intensities in different patient groups were performed by Mann-Whitney *U* test, and statistical significance was determined by $P < 0.05$. Numbers at the bottom of the table show the numbers of patients/individuals studied. MM, molecular mass.

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. Tables 1 and 2 summarize the clinical parameters and biomarker peak intensities in all 42 NPC patients under study. To define more precisely the peak elevations of the two biomarkers in relapsed patients, we set a cutoff value for each biomarker as the average highest peak intensity in patients during remission plus 2 SDs. According to the two cutoff values (3.7 and 3.6 for 11.6 and 11.8 kDa, respectively), 22 (71%) and 21 (68%) of the 31 patients at relapse showed elevations of the two biomarkers, respectively, whereas none of the 11 patients (0%) at remission had elevation in either biomarker. In contrast, only 1 of 20 (5%) relapsed patients at diagnosis had elevation. Thirty-nine sera collected at diagnosis from lung cancer control patients also had significant biomarker elevations (14.4 ± 20.2 and 16.3 ± 22.9 , respectively), as compared with the normal individuals ($P < 0.001$) and the NPC patients at diagnosis ($P < 0.001$). When applying the same cutoff criterion but using normal individuals

as negative controls (cutoff values of 0.54 for 11.6 kDa and 0.47 for 11.8 kDa), we observed that 16 of 39 (41%) of lung cancer patients also showed elevation.

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. 3A. 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,⁴ 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.*, 1.0×10^4 ; 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 another 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

Table 1 Clinical parameters and peak intensities of the 11.6- and 11.8-kDa biomarkers in the relapse group of nasopharyngeal cancer patients under study

Patient No.	Sex/Age (yr)	Stages ^a		Sites of recurrence ^b	Chemo-response ^c	Peak intensities of 2 biomarkers			
		Overall	TNM ^d			At diagnosis		At Rec/Prog	
						11.6 kDa	11.8 kDa	11.6 kDa	11.8 kDa
1	M/56	4	T ₂ N ₃ M ₀	Bilat Ax LN Met	PR	NA	NA	56	43.4
2	M/60	3	T _{23ab} N ₁ M ₀	NP and BOS Rec	PR	5.2	11.6	51.5	57.4
3	M/31	3	T ₂ N ₂ M ₀	Bone, Liver, and Spleen Met	PR	1	1	20.9	21.6
4	M/37	4	T ₂ N ₃ M ₀	NP and Neck LN Rec; Liver, Abd LN, Lung and Bone Met	PR	1.1	1.3	11.5	6.5
5	M/56	3	T ₂ N ₂ M ₀	Lung and Med LN Met	CR	NA	NA	50.3	63.2
6	M/47	4	T ₂ N ₃ M ₀	Liver, Pleur, and Groin LN Met	SD	0.4	0.6	32.3	38.7
7	M/47	3	T _{23ab} N ₀ M ₀	NP and Neck LN Rec	SD	0.6	0.7	0.7	0.8
8	F/60	3	T _{3abd} N ₀ M ₀	Ax and Infclav LN Met	CR	1.3	2	4.5	7.5
9	M/51	4	T ₂ N ₃ M ₀	Neck LN Rec; Liver, Lung, and Abd LN Met	PR	NA	NA	51	70.1
10	M/55	3	T ₂ N ₂ M ₀	Liver and Abd LN Met	PR	NA	NA	8.6	11.4
11	M/45	2	T ₂ N ₀ M ₀	NP Rec, Lung Met	CR	NA	NA	1.5	2
12	M/45	4	T _{23abc} N ₂ M ₀	Bone, Lung and Liver Met	NA	0.2	0.2	8.8	5.8
13	M/49	3	T _{23ab} N ₂ M ₀	Lung Met	PR	0.8	1.3	34.2	37.3
14	M/40	3	T _{23abd} N ₂ M ₀	NP and BOS Rec	PR	0.5	0.6	0.2	0.3
15	M/51	3	T ₂ N ₂ M ₀	Bone Met	NA	NA	NA	71.8	71.8
16	M/41	3	T _{23abd} N ₀	NP and BOS Rec	SD	0.8	1	5	5.7
17	M/61	2	T ₁ N ₁ M ₀	Abd LN Met	PR	NA	NA	1.1	0.8
18	M/63	3	T _{23b} N ₁ M ₀	NP and BOS Rec	PR	NA	NA	0.7	1.4
19	M/50	3	T _{23a} N ₂ M ₀	Lung and Pleur Met	PR	NA	NA	89.8	97
20	M/43	5	T ₂ N ₃ M ₁	NP and BOS Rec; Lung and Pleur Met	PR	0.4	0.6	7.9	6.6
21	M/46	3	T _{23ab} N ₂ M ₀	Lung Met	SD	0.2	0.4	11.8	17.1
22	M/51	2	T ₂ N ₁ M ₀	NP and BOS Rec	CR	0.5	0.5	5.8	6.1
23	M/38	3	T ₂ T _{3ab} N ₀ N ₀	Lung and Med LN Met	CR	0.6	0.8	13.7	17.9
24	F/47	3	T ₂ T _{3ab} N ₁ M ₀	Lung Met	PR	0.1	0.1	0.7	0.9
25	M/42	2	T ₂ N ₁ M ₀	Liver Met	CR	0.4	0.6	0.3	0.4
26	M/64	3	T ₂ N ₂ M ₀	Lung, Liver and Bone Met	PR	NA	NA	30	30.1
27	M/59	4	T ₂ N ₂ M ₀	Neck LN Rec; Liver, Lung, Bone, Med and Abd LN Met	CR	NA	NA	61	62.1
28	F/34	3	T _{23abd} N ₂ M ₀	NP and BOS Rec; Lung, Bone and Abd LN Met	PR	0.2	0.2	11.2	10.2
29	M/34	3	T _{23abc} N ₀ M ₀	NP and BOS Rec	SD	0.2	0.3	0.3	0.3
30	M/50	3	T _{23abd} N ₀ M ₀	Lung and Pleur Met	PR	0.1	0.2	0.6	0.5
31	M/38	3	T _{23abcd} N ₁ M ₀	Lung Met	PR	0.5	0.7	7.4	3.4

^a Staging classification according to the staging system of Ho (18).

^b 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.

^c Chemo-response: CR, complete response; PR, partial response; SD, static disease; NA, not available.

^d TNM, Tumor-Node-Metastasis; Rec/Prog, recurrence or progression of disease.

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 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

Patient No.	Sex/Age (yr)	Stages ^a		Follow-up period (mo)	Peak intensities of two biomarkers			
		Overall	TNM ^b		At diagnosis		At follow up	
					11.6 kDa	11.8 kDa	11.6 kDa	11.8 kDa
32	F/35	2	T ₂ N ₀ M ₀	96	1	1	2.3	2
33	M/59	3	T _{23b} N ₁ M ₀	100	0.8	0.9	1.2	1.4
34	F/51	2	T ₂ N ₁ M ₀	102	0.04	0.1	0.4	0.4
35	M/40	3	T ₁ N ₂ M ₀	114	0.3	0.4	1	1
36	M/44	3	T ₁ N ₂ M ₀	104	0.8	1	1	1.1
37	M/42	2	T ₂ N ₁ M ₀	97	1.3	2.5	1.3	2.5
38	M/52	2	T ₂ N ₀ M ₀	105	0.2	0.2	3	3
39	M/44	3	T _{23ab} N ₀ M ₀	112	0.4	0.6	3	2.3
40	F/43	3	T ₂ N ₂ M ₀	113	0.1	0.1	0.4	0.5
41	F/45	3	T ₂ N ₂ M ₀	98	0.5	0.7	3.2	3
42	M/48	2	T ₂ N ₀ M ₀	105	0.4	0.5	1.2	0.9

^a Staging classification according to the staging system of Ho (18).

^b TNM, Tumor-Node-Metastasis.

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 algo-

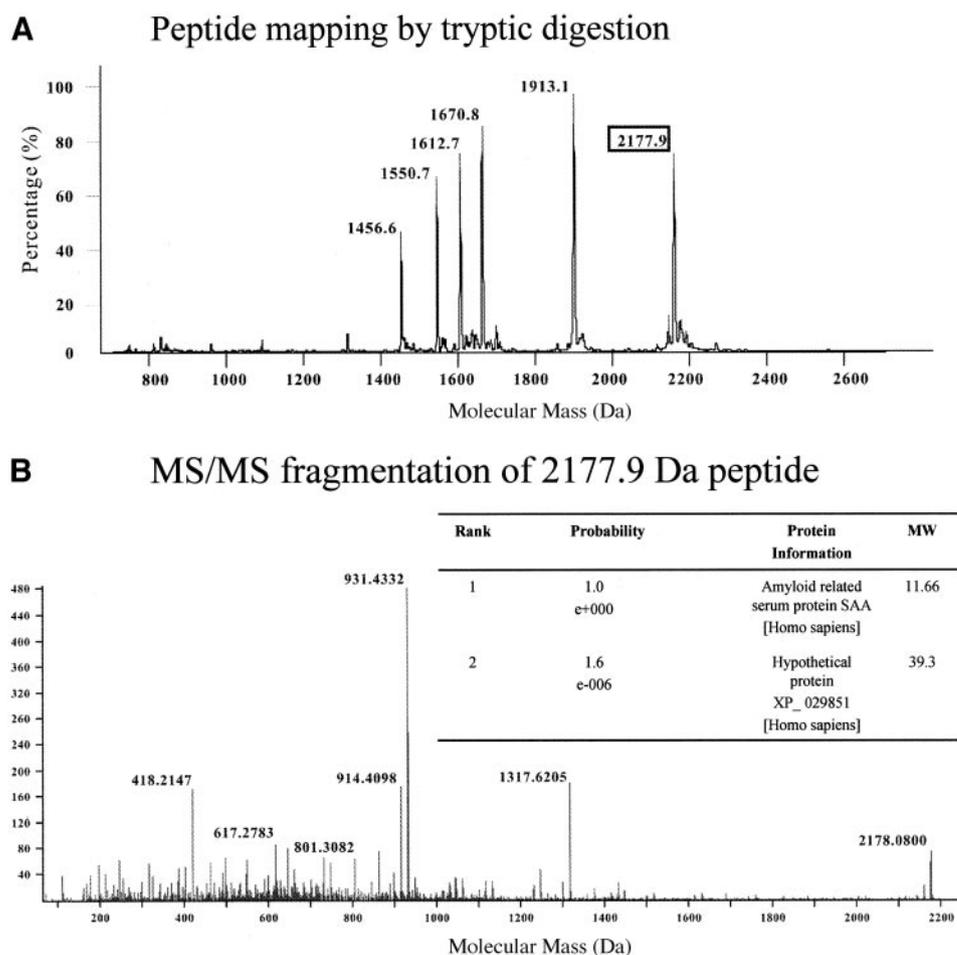
riothm, which could sensitively and specifically identify even early stages of ovarian cancer, was discovered (8, 30).

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 various isoforms in a molecular mass range of 11–14. It is derived mainly from two genes, *SAA1* and *SAA2* (31), mapped at chromosome 11p15.4–15.1 (32), with the other genes being 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-

Fig. 3 Protein identification.

A, peptide mapping of the two relapse-associated biomarkers by tryptic digestion. B, tandem mass spectrometry (MS/MS) fragmentation analysis of 2177.9-Da peptide generated from tryptic digest of the two biomarkers. The two bands at 11.6 and 11.8 kDa were cut from the polyacrylamide gel and partial tryptic digestion was performed as described in "Materials and Methods." A shows the peptide map after digestion. One of the major peptides at 2177.9 Da generated from the tryptic digest was further subjected to MS/MS fragmentation. The MS/MS ion fingerprint data as shown in B were entered into the Mascot search engine (Internet address: www.matrixscience.com/home.html) to match with those from the proteins in search engine database (Internet address: prowl-rockefeller.edu) to find the identity of protein (see "Materials and Methods"). *Inset table in B* shows identification of the proteins as human serum amyloid A protein (SAA) with 100% (1.0×10^0) probability of match in 34 of 37 ions in the fingerprint, thus giving serum amyloid A protein the first ranking protein matched.



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

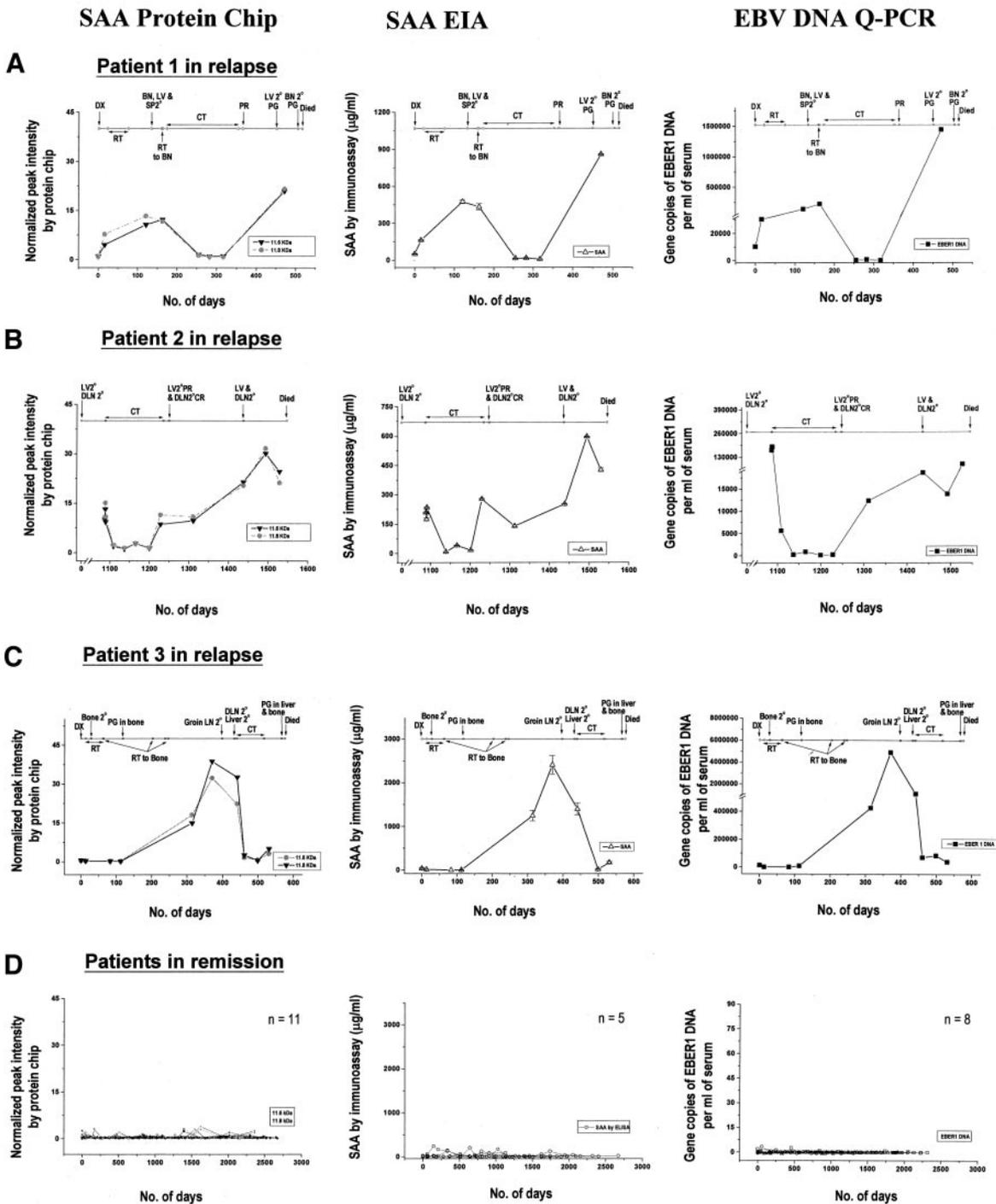


Fig. 4 Longitudinal monitoring of serum amyloid A (SAA) protein level by protein chip profiling and immunoassay and circulating serum Epstein-Barr virus (EBV) DNA by real-time quantitative PCR (Q-PCR) in nasopharyngeal carcinoma (NPC) patients. *A*, *B*, and *C*, three NPC patients in relapse were monitored 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; △, follow-up curves of SAA protein by immunoassay; ■ (EBER1), follow-up of serum EBV DNA encoding EBV small RNA-1 by Q-PCR; *BN 2°*, bone metastasis; *CR*, complete response to chemotherapy; *CT*, salvage chemotherapy; *DLN 2°*, distant lymph node metastasis; *DX*, histopathological diagnosis of NPC; *Groin LN 2°*, metastasis in lymph nodes of the groin; *LV 2°*, liver metastasis; *LV 2°CR*, partial response to chemotherapy in tumor lesion in metastatic liver; *DLN 2°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 2°*, spleen metastasis.

(43). 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%.⁵ A manuscript is under preparation to report this finding.⁶ 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.

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⁵ Unpublished data.

⁶ Manuscript in preparation.

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