

Serum proteome analysis for profiling protein markers associated with carcinogenesis and lymph node metastasis in nasopharyngeal carcinoma

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Received: 8 December 2007 / Accepted: 16 February 2008 / Published online: 21 March 2008
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Abstract Nasopharyngeal carcinoma (NPC), one of the most common cancers in population with Chinese or Asian progeny, poses a serious health problem for southern China. It is unfortunate that most NPC victims have had lymph node metastasis (LNM) when first diagnosed. We believe that the 2D based serum proteome analysis can be useful in discovering new biomarkers that may aid in the diagnosis and therapy of NPC patients. To filter the tumor specific antigen markers of NPC, sera from 42 healthy volunteers, 27 non-LNM NPC patients and 37 LNM NPC patients were selected for screening study using 2D combined with MS. Pretreatment strategy, including sonication, albumin and immunoglobulin G (IgG) depletion, was adopted for screening differentially expressed proteins of low abundance in serum. By 2D image analysis and MALDI-TOF-MS identification, twenty-three protein spots

were differentially expressed. Three of them were further validated in the sera using enzyme-linked immunosorbent assay (ELISA). Our research demonstrates that HSP70, ICAM-1 and SAA, confirmed with ELISA at sera and immunohistochemistry, are potential NPC metastasis-specific serum biomarkers which may be of great underlying significance in clinical detection and management of NPC.

Keywords Nasopharyngeal carcinoma · Serum proteome · Carcinogenesis · Lymph node metastasis

Abbreviations

NPC	Nasopharyngeal carcinoma
2D	Two-dimensional electrophoresis
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry
LNM	Lymph node metastasis
ICAM-1	Intercellular adhesion molecular-1
HSP70	Heat shock protein 70
SAA	Serum amyloid A1 protein
ELISA	Enzyme-linked immunosorbent assay
IHC	Immunohistochemistry

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Introduction

Nasopharyngeal carcinoma (NPC) is a rare malignancy in most parts of the world, though it is highly prevalent in Southern Asia, where the incidence is about a 100-fold higher than in other populations. It is one of the most confusing, commonly misdiagnosed and poorly understood diseases. Previous studies show that the cancer is an Epstein-Barr virus-associated malignancy with a

remarkable racial and geographical distribution. The etiology of NPC is thought to be associated with a complex interaction of genetic, viral, environmental and dietary factors. Thanks to the advancements in genomics, proteomics and bioinformatics in recent decades, the etiology, carcinogenesis and progression of the disease is better understood. Research into these components may unravel the pathways in NPC development and potentially decipher the molecular characteristics of the malignancy [1, 2]. NPC is an insidious tumor and is usually at the stage of metastasis involving lymph nodes or other organs before it can be found. Thus early and accurate diagnosis is very important for therapy and prognosis of NPC patients. Unfortunately, no effective method of accurately diagnosing new-onset NPC is available now. We presume if specific serum biomarkers associated with NPC metastasis can be identified on the basis of advancements in genomics, proteomics and bioinformatics, an approach to the early detection and monitoring of NPC may be found.

2D comparative proteome analysis is a new technology for the separation and identification of disease-specific proteins, and it has been applied successfully to screen potential biomarkers for NPC in cell lines and tumor tissues [3–8]. In our previous studies, we employed proteomic techniques to study protein changes of CNE2, a poorly differentiated squamous carcinoma cell line of human NPC cells, induced by 12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA). It is likely that TPA promotes NPC in necessary cooperation with EBV, or that it may function as an anti-proliferative or differentiative revulsant in noninitiated cells [4]. However, if a marker can be detected only in surgical specimens, its clinical significance is limited, especially for early screening or diagnosis. Recently, Saeid R. et al. reported their pioneering work on NPC serum analysis by 2D without any pretreatment, revealing the enhanced expression of relatively abundant proteins as ceruloplasmin (CPL) [9]. In order to find relatively low-abundant serum proteins which may be more valuable in predicting NPC progression, we pretreated serum with sonication, albumin and IgG depletion before 2D analysis. By comparing 2D image analyses for healthy volunteers, non-LNM NPC and LNM NPC patients, we may identify the differentially expressed proteins so that specific serum biomarkers associated with NPC metastasis can be found.

Materials and methods

Subjects

Serum samples from 42 healthy volunteers and 64 NPC patients were available for comparative proteomic analysis. Samples from NPC patients were obtained before any

clinical treatment. All the patients had a nasopharyngeal biopsy and NPC was confirmed by two independent pathologists unaware of the patient's condition. Careful medical examinations and CT scan determined non-LNM cases and LNM cases in the NPC patients. Clinical data of all the subjects for 2D and ELISA are shown in Table 1. In the screening study, serum samples from the same group were pooled to smooth intrinsic individual differences and enhance common characteristic traits only related to disease status [10], aliquoted, and stored at -80°C before analysis. In the validation study, 26 of the patients received a mean of 17.8 months of follow-up (range, 6–22 months) after radiotherapy and/or chemotherapy. Six of the followed-up patients died of NPC, and the other survived the follow-up period.

Depletion of high-abundance proteins

Sigma-Aldrich ProteoPrep[®] Immunoaffinity Albumin and IgG Depletion Kit was used for depletion of proteins, albumin and IgGs, allowing visualization of co-migrating proteins with albumin and IgG on a 2D gel and also higher sample loads for improved visualization of lower abundance proteins. A total of 30 μl of serum was diluted with 100 μl of equilibration buffer from the depletion kit (Sigma) and sonicated using four ultrasonic bursts of 15 s each. Between sonications, the samples were chilled on ice. Then serum albumin and IgG was depleted according to the manufacturer's instructions. After collection of the bound proteins, the majority (>95%) of the unbound proteins were left in the depleted serum sample. Modified Bradford assay was used to measure the protein concentrations of all serum samples [11].

2D analysis

The Immobiline Dry strip (pH 4–7, length 17 cm, Bio-Rad) was rehydrated with 600 mg protein in 350 ml rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 5 mM tributylphosphine, 1% IPG buffer and 0.002% bromophenol blue for 14 h at room temperature. IEF was performed using Protean IEF cell (Bio-Rad, Hercules, CA, USA) with a total of 70 kVh. Then the strip was subjected to two-step equilibration in equilibration buffer containing 6 M urea, 30% glycerol, 2% SDS and 50 mM Tris-HCl (pH 6.8) with 1% w/v DTT for the first step, and 2.5% w/v iodoacetamide for the second step. The second-dimensional SDS-PAGE gel (13% T, 2.7% C, and $20 \times 20 \times 1 \text{ mm}^3$) was carried out using a Protean II xi 2D cell (Bio-Rad) according to the following procedure: 30 min at a constant current of 12 mA followed by 24 mA per gel until the bromophenol blue front reached the bottom of the gel. Subsequently the gels were stained with

Table 1 Serum samples in the study

	Samples for 2-DE			Samples for ELISA		
	Healthy	NPC	Positive	Healthy	NPC	Positive
Lymph node status	/	Negative	Positive	/	Negative	Positive
Number	42	27	37	30	27	30
Gender (Male/Female)	22/20	21/6	23/14	15/15	21/6	20/10
Age (\pm SD)	42 \pm 7.9	43 \pm 10.9	50.5 \pm 11.7	42.5 \pm 8.9	43 \pm 10.9	47.6 \pm 10.2

MS accommodated silver staining [12]. 2D of each sample was run in triplicate to minimize run-to-run variation. The silver-stained 2D gel were scanned using a Power-Look 1100 imaging scanner (Umax, Dallas, TX, USA) and analyzed using PDQuest 7.1 software package (Bio-Rad).

In-gel enzymatic digestion

Protein spots were excised from gel with an operating knife blade, destained twice with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (1:1 v/v) and then equilibrated in 50 mM NH_4HCO_3 to pH 8.0. After dehydrating with ACN and drying in N_2 at 37°C for 20 min, the gel pieces were rehydrated in 15 ml trypsin solution (10 $\mu\text{g}/\text{ml}$ in 25 mM NH_4HCO_3) at 4°C for 30 min and incubated at 37°C overnight. Peptides were extracted twice using 0.1% TFA in 50% CAN and dried with N_2 .

MALDI-TOF-MS identification

The peptide mixtures were solubilized with 0.5% TFA, with saturated α -cyano-4-hydroxy-trans-cinnamic (CHCA) solution in 0.1% TFA/50% acetonitrile as the matrix and analyzed using M@LDI R (Micromass, Manchester, UK). Mass spectra were externally calibrated with lock mass 2,465.199 Da and internally calibrated with autodigested peaks of trypsin (MH⁺: 2,211.105 Da).

Protein identification and database searching

Protein identification using peptide mass fingerprinting (PMF) was performed by the MASCOT search engine (<http://www.matrixscience.com/>, MatrixScience Ltd., London, UK) against the MSDB protein database. The errors in peptide masses were in the range of 25 ppm. One missed tryptic cleavage site per peptide was allowed during the search. Proteins matching more than four peptides and with a MASCOT score higher than 64 were considered significant ($P < 0.05$). Carboamidomethylation of cysteine was selected as the static modification and oxidation of methionine as the differential modification. Protein identification results were filtered with peakErazor software.

Enzyme-linked immunosorbent assay (ELISA) confirmation

ELISAs were conducted to confirm the protein identification and differential expression of sICAM-1, SAA and HSP70 in sera from the three groups. Measurements were done by commercially available ELISA kits (StressXpress Hsp70 ELISA Kit: EKS-700B, Stressgen Biotechnologies, Victoria; High sensitivity sICAM-1 ELISA Kit: BMS241, Bender Medsystems Company, Australian; Human SAA ELISA Kit: EL10015, YES Biotech Laboratories Ltd, Canada), in accordance with the manufacturer's instructions. All sera were stored at -80°C before they were measured. Both standards and samples were run in duplicate. The main protocols were as follows: monoclonal coating antibody was adsorbed onto microwells; target protein present in the sample or standard bound to antibodies was adsorbed to the microwells; second antibody was added and bound to target protein captured by the first antibody; following incubation unbound enzyme was removed during a wash step; a color reaction was formed and absorbance was measured at 450 nm. The standard curve was used to determine the concentration of target protein in an unknown sample.

Immunohistochemistry (IHC) assay

The previous studies showed that sICAM-1 in serum could be released by membrane-associated ICAM-1 of cancer cell [13, 14]. However, SAA is synthesized and secreted into serum by hepatocytes in the liver during the acute phase [15, 16]. In the present study, therefore, expressions of only HSP70 and ICAM-1 were detected in tissue by IHC assay. Three-micrometer sections of paraffin-embedded tissues were cut for a prior antigen retrieval step in boiling 10 mM citrate buffer pH 6 for 20 min. The fixed tissues were subjected to immunostaining using an ultrasensitive S-P technique (MaiXin, China). Briefly, the slides were incubated overnight at 4°C with goat anti-ICAM and anti-HSP70 polyclonal antibody (1:200) (Wuhan Boster Biological Technology Co., Ltd., China). The concentrations of the primary antibodies used have been optimized previously. After washing with PBS three times, they were

treated with biotin-conjugated second antibody before adding streptavidin-peroxidase. For color reaction, diaminobenzidine (DAB) was used. All series included positive controls. For negative controls, the antibody was replaced by PBS. To evaluate the expression, the cellular localization of immunoreactivity was determined and scored for both intensity (negative, weak, moderate and strong) and proportion (5–25, 26–50, 51–75 and 76–100%) of stained cells. To allow comparison of immunostaining with pathological stage and metastasis for each cancer, integer values were assigned to the scores of intensity (0–3) and proportion of tumor cells stained (0–4). If the evaluations did not agree, the specimens were reevaluated and then classified according to the assessments given, most frequently, by the observers. The proportion and intensity scores were then added to obtain a total score, which was labeled as one of the four following categories: (a) high positive (+++), total score of 6–7; (b) moderate positive (++), total score of 4–5; (c) low positive (+), total score of 2–3; (d) negative (–), total score of 0–2.

Statistical analysis

All the analyses performed were descriptive. The serum levels of protein expressions were compared using one-way ANOVA test. Associations between immunohistochemical scores and clinicopathological variables of tissue specimens were evaluated by Fisher's exact test or χ^2 test. PDQuest software was used to establish a master gel representing each group [17]. For differential expression of matched gels, protein spots whose intensities were either increased or decreased two-fold or greater were marked and then confirmed by manual inspection of all relevant 2D gel, not only those included in the matchsets, to ensure consistency. Quantitative analysis was performed using the Student's *t*-test among three groups of gels. All statistical analysis was performed with SPSS 10.0 software and *P* value <0.05 were considered statistically significant.

Results

Quantitative comparison and identification of protein spots on 2D gels

Among the three groups, we found twenty-three spots significantly altered ($P < 0.05$) or absent/emergent, 13 of which were identified successfully (Fig. 1). The corresponding detailed differential expression patterns are shown in Fig. 2a. Quantitative comparison and MS identification of the 13 differentially expressed proteins are shown in Fig. 2b and summarized in Table 2, respectively. Overall, transferrin (TRF) and transthyretin (TTR) were

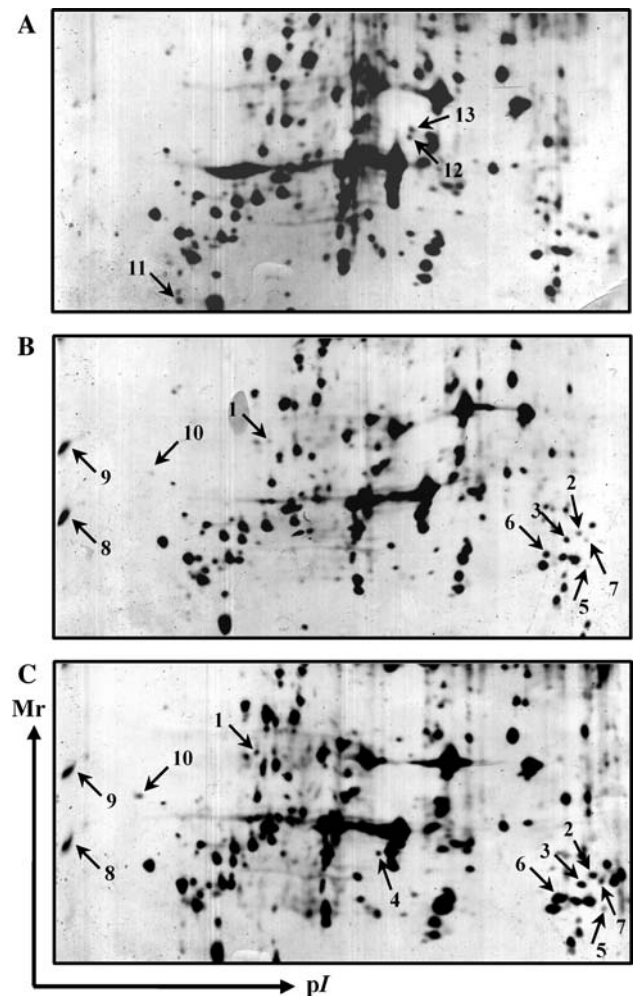


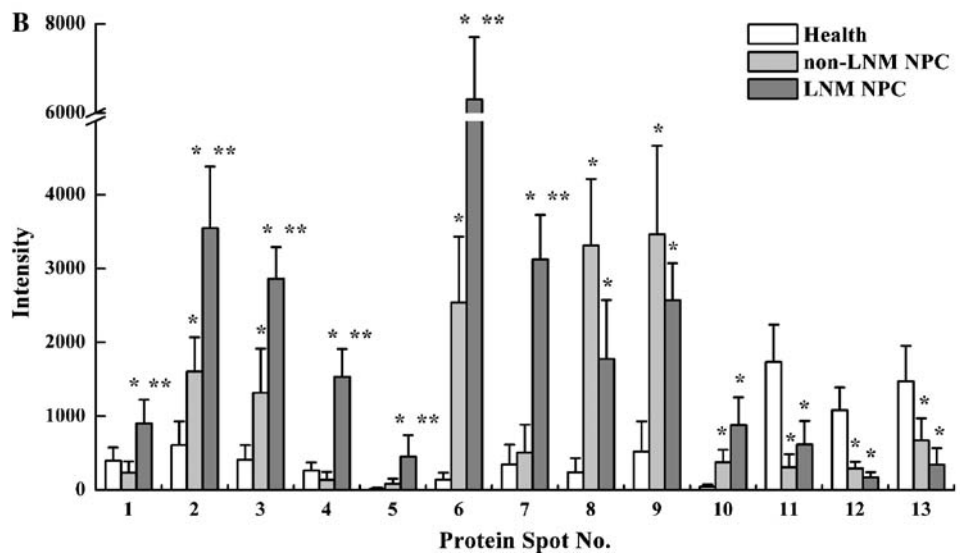
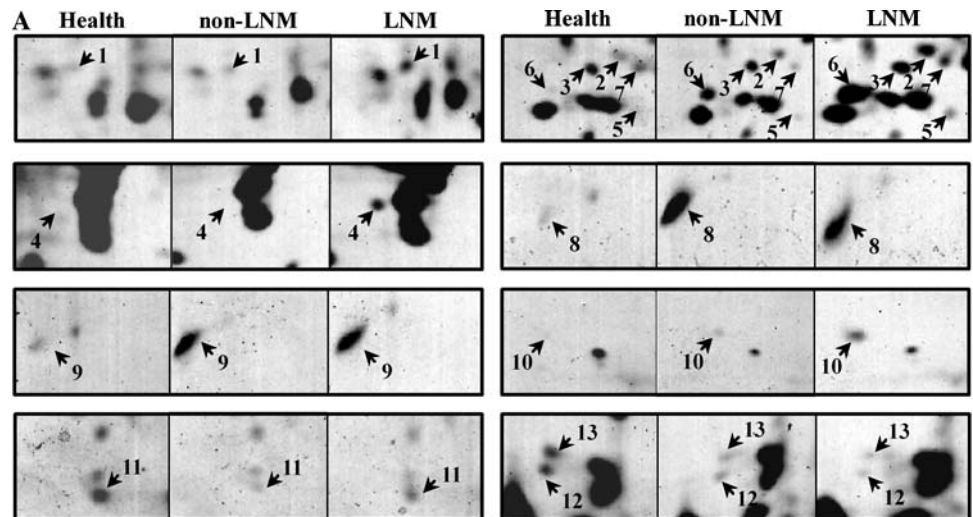
Fig. 1 Representative 2D patterns of albumin and IgG depleted serum from healthy (a), non-LNM NPC (b) and LNM NPC groups (c). In the 2D images, spots '1–10' indicate up-regulation and '11–13' down-regulation in NPC group. Black arrows indicate protein spots that were identified successfully by MS

down-regulated and 12-lipoxygenase (12-LOX), serum amyloid A1 protein precursor (SAA), soluble intercellular adhesion molecule-1 (sICAM-1) and lysine-specific histone demethylase1 (LSD1) were up-regulated in two NPC groups compared with the healthy group. Furthermore, increased expression levels of 12-LOX, sICAM-1 and SAA were observed in LNM NPC as compared with non-LNM NPC. However, heat shock protein 70 (HSP70) and cytochrome P450 (CYP450) were expressed significantly and constantly only in LNM NPC patients. The MALDI-TOF-MS mass spectra are shown in Fig. 3.

ELISA confirmation

Figures 4 and 5 show the ELISA results of sICAM-1, SAA and HSP70 performed in the three groups. There were

Fig. 2 Detailed 2D patterns of the thirteen differentially expressed proteins among healthy, non-LNM and LNM NPC groups (a). Corresponding gel positions are shown in Fig. 1. Magnified 2D maps demonstrating the different expressions of selected proteins are listed in Table 2. The densitometric analysis of each protein was calculated from nine different gels using PDQuest 7.1 software package (b). Each bar represents the mean \pm S.D. of intensity, with significant differences between groups found by two-tailed student's *t*-test. Note that this panel has a different vertical (y) axis and a break in the axis to accommodate the poor performance of the subject spot No.6. The break region is from 5000 to 6000. **P* < 0.05, compared with healthy group; ***P* < 0.05, compared with non-LNM NPC group



statistically significant differences between health and NPC groups ($P < 0.01$) as well as between non-LNM and LNM NPC groups ($P < 0.05$) (Fig. 4). And with the TNM stage advanced, the serum levels of sICAM-1 and HSP70, were detected significantly elevated, but that of SAA not (Fig. 5). Furthermore, bivariate correlation analysis showed a significant relationship between the serum levels of the three proteins ($P < 0.01$). The follow-up showed that the serum levels of sICAM-1 and HSP70, but not of SAA, were strongly associated with NPC mortality (Student's *t*-test, $P = 0.001$, 0.001 and 0.132 , respectively). Our ELISA findings were consistent with results of 2D and silver-staining.

IHC assay

In NPC patients, HSP70 and ICAM-1 were observed in tumor cells but not in stromal, endothelial or

inflammatory cells. The immunoreactivity was located in both membrane and cytoplasm for ICAM-1 and only in cytoplasm for HSP70 (Fig. 6). The immunoreactivities of HSP70 and ICAM-1 were observed in 37 (64.9%) and 43 (75.4 %) of the 57 cases of NPC, respectively. The correlation of positive rates showing HSP70 and ICAM-1 immunoreactivity with clinicopathological characteristics of the cases is summarized in Table 3. Statistically, positive expression rates of HSP70 and ICAM-1 were significantly correlated with lymph node metastasis (LNM) of NPC. Furthermore, we found that positive expression rates of ICAM-1 (6/6, 100%) and HSP70 (5/6, 83.3%) were high in the cases that died of NPC during the follow-up period ($P > 0.05$). The results manifested that ICAM-1 and HSP70 expressions were strongly associated with NPC metastasis and mortality, indicating poor prognosis.

Table 2 Identification results of differentially expressed proteins among healthy volunteers, non-LNM NPC and LNM NPC patients

No. ^a	Protein description	MSDB ID	Protein level ^b	MASCOT score ^c	Sequence coverage ^d (%)	Theoretical Mr/pI ^e
1	Ovochymase precursor	Q7RTY7	↑	116	52	124,947/8.67
2	Arachidonate 12-lipoxygenase	AAA51587	↑	193	68	75,694/5.82
3	sICAM-1	Q99930	↑	112	54	4270/6.40
4	Cytochrome P450	Q53EX9	↑	207	57	55,635/6.83
5	Serum amyloid A1 protein precursor	YLHUS	↑	170	85	13,524/6.28
6	Hemoglobin beta subunits are s-nitrosylated	1BUWB	↑	207	55	15,565/6.76
7	E1A 10S protein	Q9YLA2	↑	143	69	28,493/4.09
8	Lysine-specific histone demethylase 1	O60341	↑	172	58	93,358/6.11
9	KIAA1622 protein	Q9HCF0	↑	78	61	102,351/8.42
10	Heat shock 70 kDa protein (HSP70)	Q6G1F9	↑	157	53	68,161/4.88
11	Transferrin	Q9NQB8	↓	167	67	77,050/6.81
12	UPF0366 protein C11orf67	AAD40378	↓	104	58	12,798/8.58
13	Transthyretin	AAA61181	↓	87	69	12,836/5.35

^a The numbers indicate the spot positions in 2D gel as shown in Figs. 1 and 2

^b ↑, up-regulation in NPC patients; ↓, down-regulation in NPC patients

^c By MALDI-TOF MS analysis

^d Calculated by amino acid count

^e Calculated from the database entry without any processing

Discussion

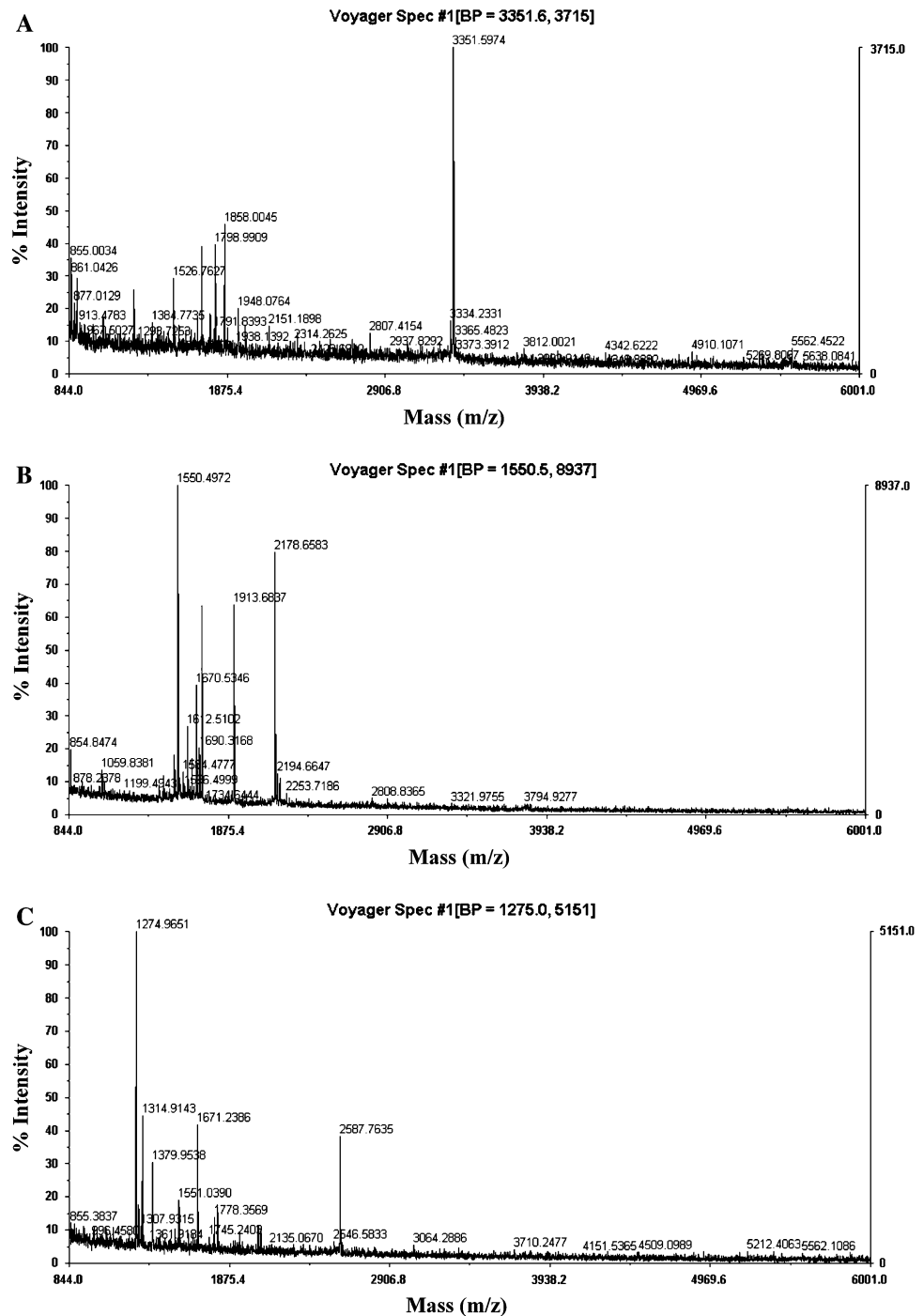
Serum is a complex body fluid, containing a large diversity of proteins. More than 10,000 different proteins are present in the human serum and many of them are secreted or shed by cells during different physiology or pathology processes [18]. Consequently, proteomics has raised great expectations for the discovery of biomarkers to improve diagnosis or classification of a wide range of diseases, including cancers [19]. Serum is expected to be an excellent source of protein biomarkers because it circulates through, or comes in contact with, all tissues. During this contact it is likely to pick up proteins secreted or shed by tissues, which has recently been tested and confirmed [20]. However, serum has been termed as the most complex human proteome [21] with considerable differences in the concentrations of individual proteins, ranging from several milligrams to less than one pictogram per milliliter [22]. Another analytical challenge for biomarker discovery arises from the high variability in the concentration and state of modification of some human plasma proteins between different individuals [23]. Despite these limitations, human serum holds immense diagnostic potential. In the last decade, several large-scale projects have been initiated, aimed at characterizing the human plasma/serum proteome.

In the mean time, several serum proteomic studies on NPC have been reported. Cho et al. performed protein chip

profiling analysis with surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) technology on sera from NPC patients and demonstrated that SAA may be a potentially usefully biomarker for NPC [24]. However, the technology for discovery of new cancer biomarkers has recently been questioned for its flaws, such as its qualitative nature, high identification error rate, poor reproducibility and nonspecific absorption matrices [25–27]. 2D-based comparative proteome analysis, though a new technology for the separation and identification of disease-specific proteins, has been applied successfully to screen potential biomarkers for NPC in cell lines and tumor tissues [3–8].

Recently, Saeid et al. reported their pioneering work on NPC serum analysis by 2D, revealing the enhanced expression of such relatively abundant proteins as ceruloplasmin (CPL) [9]. However, in 2D study, abundant proteins, such as albumin and IgG, that account for approximately 60–97% of the total serum proteins [28], mask other proteins that migrate to the surrounding areas and limit the loading amount of serum. As albumin and IgG are known to function as carriers and transporters of important proteins such as hormones, cytokines, and lipoproteins within the blood [29–31], the depletion of these two highly abundant proteins may result in the loss of potentially important proteins bound to them at the same time. In order to release those adsorbed or bound proteins,

Fig. 3 PMF of protein spots No. 3, 5 and 10, respectively, representing sICAM-1 (panel A), SAA (panel B), and HSP70 (panel C), which were expressed differentially between the groups of non-LNM and LNM NPC (MASCOT scores are 65, 85 and 78, respectively, $P < 0.05$)



we sonicated the diluted sera before the depletion and desalting steps as suggested by Quero et al. [32, 33]. The improvements we made in sample preparation enabled us to find some valuable low abundant proteins.

Of the 13 successfully identified protein spots, we focused on 10 up-regulated proteins in NPC for further validation. We also accumulated certain knowledge about three proteins by literature profiling. Finally, we decided to further investigate sICAM-1, HSP70 and SAA, which

seemed more associated with our research interest, using both ELISA and IHC to validate their differential expressions. Intriguingly, most of these identified proteins have been reported to be associated with carcinogenesis and tumor metastasis. Our research is herein chiefly concerned with the functional implications of the three proteins to NPC at the serum level.

Although the source of sICAM-1 has not been fully elucidated, researches show it can be released by cancer

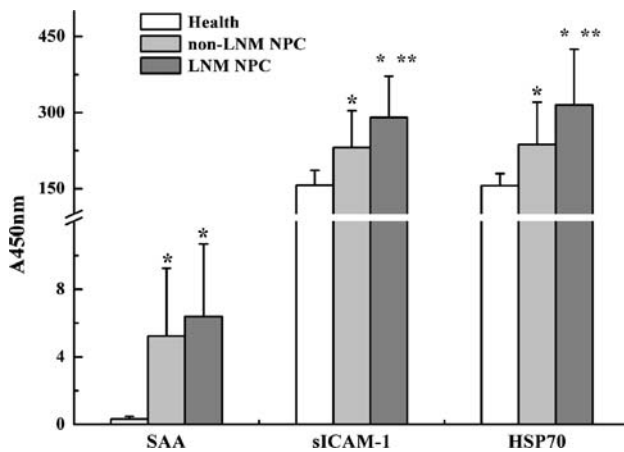


Fig. 4 Quantitative comparison of sICAM-1, HSP70 and SAA in sera among healthy, non-LNM NPC and LNM NPC groups. Each bar represents the mean \pm S.D. of the concentration, with significant differences between groups found by one-way ANOVA test. Note that this panel has a different vertical (y) axis and a break in the axis to accommodate the poor performance of the subject SAA. The break region is from 500 to 4500 $\mu\text{g/l}$. * $P < 0.05$, compared with healthy group; ** $P < 0.05$, compared with non-LNM NPC group

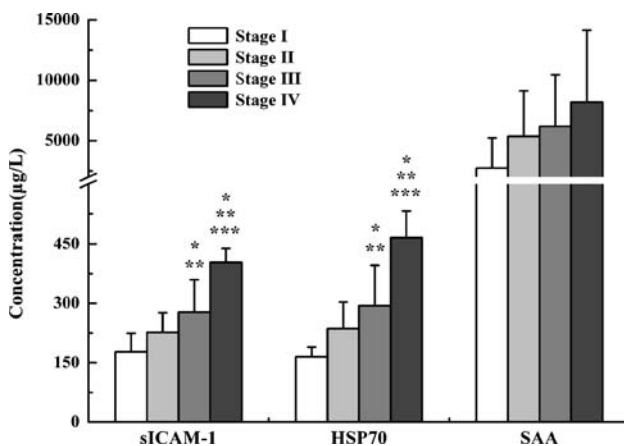


Fig. 5 Quantitative comparison of sICAM-1, HSP70 and SAA in sera among NPC patients at different TNM stage. Each bar represents the mean \pm S.D. of the concentration, with significant differences between groups found by one-way ANOVA test. Note that this panel has a different vertical (y) axis and a break in the axis to accommodate the poor performance of the subject SAA. The break region is from 600 to 2000 $\mu\text{g/l}$. * $P < 0.05$, compared with NPC patients at TNM stage I; ** $P < 0.05$, compared with NPC patients at TNM stage II; *** $P < 0.05$, compared with NPC patients at TNM stage III

cells [13, 14] as well as by peripheral blood mononuclear, endothelial, and fibroblastic cells [34]. Proteolytic cleavage of membrane-bound ICAM-1 may be the most likely mechanism for the generation of sICAM-1 [35]. In patients with certain malignancies, the serum sICAM-1 titers have been found elevated in association with tumor growth and distant metastasis of malignant melanoma [36], lung [37],

breast [38], gastric [39], hepatocellular [40], and colorectal cancers [41]. Poor survival of cancer patients correlated with a high level of serum sICAM-1 has also been demonstrated [36, 38, 39]. In our study, the positive expression of tissue ICAM-1 and levels of serum sICAM-1 were significantly correlated to the presence, progression, metastasis and mortality of NPC. sICAM-1 possesses most of the necessary extracellular structures to retain the functional activities of ICAM-1 [13, 35]. It has been reported that ICAM-1 on the surface of cancer cells or antigen presenting cells (i.e., macrophages) is a costimulatory factor that stabilizes T-cell receptor-mediated binding between these cells and T lymphocytes [42]. sICAM-1 would work as an immunosuppressive agent by blocking LFA-1 on T lymphocytes, thus rendering it less available for binding with ICAM-1 on the surface of cancer cells [43]. In this manner, the shedding of sICAM-1 may speed up the metastatic process by escaping host immune surveillance. This, probably, presents an additional potential mechanism accounting for high serum levels of sICAM-1 in NPC patients who have metastasized via hematogenous and lymphatic routes. As serum sICAM-1 may be useful for monitoring hematogenous metastasis, measuring the serum sICAM-1 level might be potentially significant in clinic.

SAA is an acute-phase protein with various isoforms in a molecular mass range of 11–14. In normal individuals, SAA is produced by hepatocytes in the liver [16]. After secreted into serum, it rapidly binds to high-density lipoprotein, with 90% of the protein particles bound [15]. A review of the literature shows that only a low level of SAA can be found in the sera of healthy individuals, despite the ubiquitous nature of SAA [44]. This is in sharp contrast to the patients with neoplastic diseases, such as those with renal [45] and colorectal [46] cancers, who showed dramatic elevation of serum SAA. The prognostic significance of SAA for other cancers has also been found by conventional radioimmunoassay, in line with the findings of this study [47]. Cho et al. demonstrated that SAA may be a potentially usefully biomarker for NPC [24]. The report fully confirmed relatively huge concentrations of SAA in serum (0.2–2 g/l) that are thousands of times higher than classical cancer biomarkers (such as CEA, PSA, CA125, etc.) originating from tumor cells. In a similar way, we further confirmed that serum SAA was much elevated in NPC patients, particularly at the process of lymph node metastases when compared with non-LNM patients. However, such biomarkers (acute-phase reactants) are not commonly considered as cancer-specific ones and expected to be elevated in other malignant diseases or inflammatory diseases as well [26, 27]. In previous reports, SAA was found elevated in different malignancies, such as cancers of kidney, colon and prostate, as well as in leukemias and

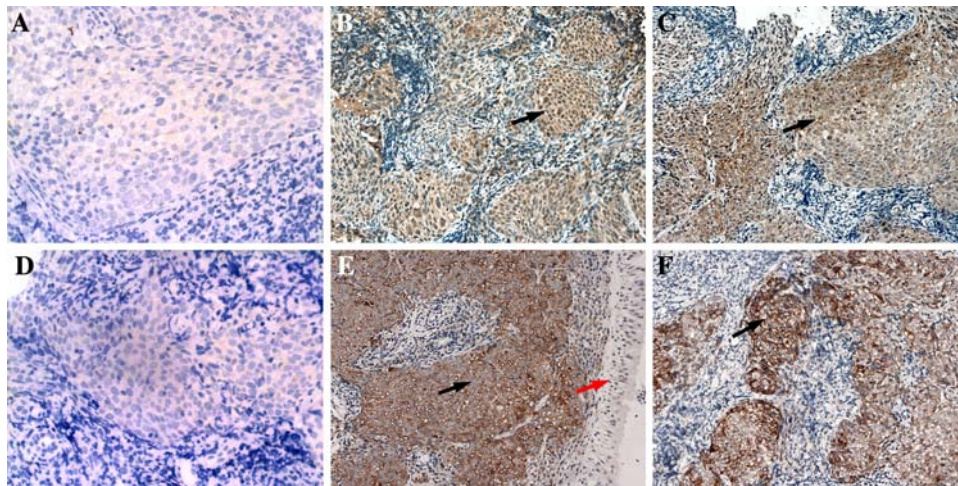


Fig. 6 Immunohistochemical detection of HSP70 and ICAM-1 in NPC (Magnification $\times 200$). The immunoreactivity was located in both membrane and cytoplasmic stainings for ICAM-1 but only in cytoplasmic staining for HSP70. The black arrows indicate the target proteins stained in brown-yellow in the NPC nest. The red arrow in E

shows negative immunoreactivity of normal nasopharyngeal epithelia. **a**, negative expression of HSP70; **b** and **c**, positive expressions of HSP70; **d**, negative expression of ICAM-1; **e** and **f**, positive expressions of ICAM-1; **a**, **b**, **d** and **e**, non-LNM NPC; **C** and **F**, LNM NPC

Table 3 Characteristics of NPC patients dependent on ICAM-1 and HSP70 expressions

Characteristics	Number	ICAM-1 expression			HSP70 expression		
		Negative	Positive (%)	<i>P</i> value	Negative	Positive (%)	<i>P</i> value
Gender							
Male	41	9	32 (78.0)	0.464	15	26 (63.4)	0.704
Female	16	5	11 (68.8)		5	11 (68.8)	
Age							
Mean (45.4)							
<45 years	26	7	19 (73.1)	0.704	9	17 (65.4)	0.945
≥ 45 years	31	7	24 (77.4)		11	20 (64.5)	
Lymph node status							
Positive	30	3	27 (90.0)	0.012	5	25 (83.3)	0.002
Negative	27	11	16 (59.3)		15	12 (44.4)	
TNM stage							
I + II	24	11	13 (54.2)	0.002	12	12 (50.0)	0.044
III + IV	33	3	30 (90.9)		8	25 (75.8)	
Survival rate							
Survival	20	6	14 (70)	0.280	6	14 (70)	1.000
Mortality	6	0	6 (100)		1	5 (83.3)	

Note: Values in parentheses are percentages

lymphomas [24, 48]. Therefore, SAA may represent a cancer epiphenomenon, unlikely to be of much clinical use in diagnosing and monitoring cancer [49], but it will be interesting to explore whether the rapid production of SAA in the liver or the epithelia of different organs [50] may be related to the stimulation by cytokines abundantly present in the NPC cells. It is also meaningful to investigate how

SAA is produced at different stages of clinical manifestation in NPC patients.

HSP70 is the main protein produced during cellular response to varied stresses, such as heat shock, ischemia/reperfusion, and oxidative changes [51, 52]. Because HSP70 confers cell protection against different stresses, it has been hypothesized that it plays a protective role in

tumor growth *in vivo* [53, 54]. Indeed, HSP70 is overexpressed in human tumors of varied origins [55], such as colorectal [56], breast [57, 58], prostate [59], liver [60] cancers and melanoma [61]. LNM and poor survival of cancer patients correlated with HSP70 overexpression has also been documented [57, 58, 61–63]. It is suggested that HSP70 is needed for *in vivo* tumor progression. In addition, HSP70 may be released from tumor cells involving involves both active secretion and passive release from necrotic cells [64]. Physiological mechanisms include co-secretion in exosomes of HSC70 with TRF and the active HSP70 secretion by the nonclassical pathway employed by cytokines [65]. Therefore, HSP70 is detectable in serum so that it could potentially be used as a biomarker for diagnosis or disease classification. Abe et al. reported that HSP70 is a marker of prostate cancer, and may be used in conjunction with PSA to identify patients with early-stage prostate cancer [59]. However, to the best of our knowledge, the association between serum level of HSP70 and NPC status has not been reported. Since our findings demonstrated that there was a modest association between serum HSP70 level and NPC staging, the serum HSP70 level should not be considered as an independent prognostic factor in NPC patients, although it might be a prognostic predictor by univariate analysis. In short, serum HSP70 may have an adjunctive clinical value in monitoring tumor progression and evaluating prognosis in NPC patients, though its clinical application as a major tumor marker is limited.

In conclusion, our serum proteomic analysis, using a comprehensive pretreatment strategy, provides a practical and exemplary tool of screening progression-associated serum proteins in NPC research. After comparing 2D image analyses for healthy volunteers, non-LNM NPC and LNM NPC patients, we successfully identified 13 differentially expressed protein spots. We further explored the differential expressions of three of the proteins, namely, sICAM-1, HSP70 and SAA, by ELISA at serum and IHC at tissue, though more work should be completed to pinpoint the direct correlation between serum level of sICAM and HSP70 and their expression level in the tissues. We suggest that the three proteins may be potential serum biomarkers which can serve as effective target points for early diagnosis and therapy of NPC patients, though further clinical research should be done before the potential come true.

Acknowledgment This work was financially supported by National Key Basic Research Program of China (973 Program) (No. 2001CB510207), Key Science and Technology Research Program of Guangdong Province (No. 2003A308401) and Natural Science Foundation of Guangdong Province (No. 5200512). We also thank Professor Liang Ping for his critical reading and revision of the manuscript.

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