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# Quantitative plasma proteome analysis reveals aberrant level of blood coagulation-related proteins in nasopharyngeal carcinoma

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ABSTRACT

Nasopharyngeal carcinoma (NPC), one of the most common cancers in Southeast Asia, is not easily diagnosed until advanced stages. To discover potential biomarkers for improving NPC diagnosis, we herein identified the aberrant plasma proteins in NPC patients. We first removed the top-seven abundant proteins from plasma samples of healthy controls and NPC patients, and then labeled the samples with different fluorescent cyanine dyes. The labeled samples were then mixed equally and fractionated with ion-exchange chromatography followed by SDS-PAGE. Proteins showing altered levels in NPC patients were identified by in-gel tryptic digestion and LC–MS/MS. When the biological roles of the 45 identified proteins were assessed via MetaCore™ analysis, the blood coagulation pathway emerged as the most significantly altered pathway in NPC plasma. Plasma kallikrein (KLKB1) and thrombin–antithrombin III complex (TAT) were chosen for evaluation as the candidate NPC biomarkers because of their involvement in blood coagulation. ELISAs confirmed the elevation of their plasma levels in NPC patients versus healthy controls. Western blot and activity assays further showed that the KLKB1 active form was significantly increased in NPC plasma. Collectively, our results identified the significant alteration of blood coagulation pathway in NPC patients, and KLKB1 and TAT may represent the potential NPC biomarkers.


## 1. Introduction

Nasopharyngeal carcinoma (NPC) is endemic in the populations of Southeast Asia [1]. Because of its radiosensitivity, the five-year survival rates of NPC patients with concurrent chemoradiotherapy could reach to 70% [2,3]. Despite a good cure probability of NPC, tumor stage at initial diagnosis remains a major survival indicator of NPC patients [4,5]. Unfortunately, most NPC patients were diagnosed at the late stages, owing to the deep location of nasopharynx and vague

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symptoms of the disease. Thus, fit-to-use biomarkers for early detection and prognosis of NPC are urgently needed [6].

Epstein–Barr virus (EBV) is highly associated with NPC [7,8]. Therefore, EBV-derived and related factors have been widely applied as the NPC markers [9,10]. Serological tests of antibodies to EBV, particularly immunoglobulin A to EBV viral capsid antigen (EBV VCA IgA), have been currently used for NPC detection, but high false-positive rates of these assays occur frequently [11]. Hence, the development of new serological markers for NPC detection remains necessary and would greatly improve NPC treatment and outcomes [6].

Advances of proteomic technologies have greatly accelerated discoveries of potential serological tumor markers [12–14]. Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (MS), that directly compares blood samples between groups, was employed in looking for disease markers [15,16]. Using this approach, a biomarker pattern consisted of four protein peaks (4097, 4180, 5912 and 8295 Da) and serum amyloid A protein have been identified for NPC detection and monitoring NPC relapse, respectively [17,18]. However, this strategy for discovering serological biomarkers is obstructed by: (i) high variation among blood specimens; (ii) substantial complexity and vast dynamic range of protein levels; and (iii) laborious protein fractionation which is essential to further reveal biomarker identities [19,20].

Using two-dimensional gel electrophoresis (2-DE) and MS, stathmin, 14-3-3 sigma and annexin I have been shown as the candidate tissue markers for NPC prognosis [21]. In addition, HSP70, sICAM-1 and SAA have also been found as the potential serum markers of NPC metastasis [22]. Although this approach remains the most classic and well-established technique for analyzing complex protein samples [23,24], it is clear that 2-DE has several disadvantages, including poor reproducibility between gels, time-consuming and labor-intensive, as well as destitute of separation for hydrophobic or highly acidic/basic proteins. As a result, 2-D difference gel electrophoresis (2-D DIGE) have been widely used as the complement to conventional 2-DE [25].

In the 2-D DIGE approach, protein samples individually labeled by mass/charge-matched and spectrally different fluorescent cyanine (Cy) dyes are mixed and then separated in one 2-D gel. Consequently, this approach reduces the experimental variations, enhances the sensitivity of protein detection and yields a more accurate quantitative analysis comparing to conventional 2-DE [26–28]. However, it still carries the disadvantages existed in 2-DE technique. To overcome these shortcomings, Dr. Hanash's group has further applied Cy dyes to the intact-protein analysis system (IPAS) for quantitatively profiling protein differences between blood samples [29,30], and discovering potential markers in lung cancer mouse model [31]. In the IPAS, blood samples depleted of abundant proteins were individually tagged with different Cy dyes, mixed equally, and fractionated sequentially in compliance with protein charge, hydrophobicity, and molecular mass. The relative protein levels were determined in the light of the Cy-dye ratios by using Amersham-developed DeCyder Software Package [29,30].

Previously, we have applied the IPAS to comparatively analyze the serum proteome of nude mice bearing with and without NPC xenografts [32]. Herein, the IPAS was used to detect the aberrant plasma proteins in the NPC patients. From biological network analysis of 45 identified proteins, plasma kallikrein (KLKB1) and thrombin–antithrombin III complex (TAT) were selected for evaluation as the potential NPC biomarkers. Subsequently, ELISA, Western blot and/or activity assays confirmed the increase of their plasma levels in NPC patients versus healthy controls. These findings demonstrate the feasibility of IPAS in differential plasma proteome analysis for NPC and indicate plasma KLKB1 and TAT as potential NPC biomarkers.

## 2. Materials and methods

### 2.1. *Patient population and clinical specimens*

All the plasma samples were collected at the Chang Gung Memorial Hospital (Taoyuan, Taiwan) between 2003 and 2007. Plasma samples for the IPAS analysis were listed in Table 1. The characteristics of plasma samples used in the ELISA, activity assay, and Western blot analysis of KLKB1 and TAT ELISA were summarized in Table 2. The study was approved by the Medical Ethics and Human Clinical Trial Committee at the Chang Gung Memorial Hospital.

### 2.2. *Removal of high abundant proteins from plasma*

Plasma was diluted 4-fold with the equilibration buffer and filtered through a 0.22-μm microcentrifuge filter tube (Agilent Technologies, DE, USA). The top seven most abundant plasma proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, and fibrinogen) were removed by multiple affinity removal system (MARS) HPLC column (4.6×100 mm; Agilent Technologies). Following depletion, the flow-through fractions were concentrated by centrifugation in Amicon Ultra-4 tubes (5-kDa molecular weight cut-off, Millipore, MA, USA). The concentrated samples were stored in aliquots at −70 °C for future use (Supplementary Fig. S1).

**Table 1 – Characteristics of plasma samples used in the IPAS analysis.**

| Plasma sample[a] | Age | TNM-T stage | TNM-N stage | Overall stage | Pathology |
|---|---|---|---|---|---|
| Healthy control | | | | | |
| HC3 | 46 | – | – | – | – |
| HC4 | 38 | – | – | – | – |
| HC5 | 45 | – | – | – | – |
| HC7 | 31 | – | – | – | – |
| HC8 | 44 | – | – | – | – |
| NPC patient[b] | | | | | |
| N115 | 28 | 1 | 1 | IIb | Non-keratinized carcinoma |
| N118 | 50 | 1 | 1 | IIb | Undifferentiated carcinoma |
| N123 | 44 | 1 | 2 | III | Non-keratinized carcinoma |
| N125 | 43 | 1 | 2 | III | Undifferentiated carcinoma |

[a] All of the healthy controls and patients are male.
[b] No distant metastasis found in these patients.