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Received January 1, 2012
Revised March 5, 2012
Accepted March 15, 2012

Research Article

Proteomic analysis reveals platelet factor 4 and beta-thromboglobulin as prognostic markers in severe acute respiratory syndrome

Previously, we reported that proteomic fingerprints were present in sera of patients with severe acute respiratory syndrome (SARS), and could separate patients into subgroups with different prognoses. In the present study, we examined the prognostic values of the SARS-associated proteomic features by biostatistical analysis, and deciphered the identities of those with prognostic values. Data of 20 SARS-associated serum proteomic features and ten serological variables from 38 SARS adult patients before treatment were subjected to multivariate logistic regression. Proteomic features of *m/z* 6634, *m/z* 7769, *m/z* 8635, and *m/z* 8865 were identified as independent prognostic markers. After purification by cation-exchange chromatography and gel electrophoresis, proteomic features of *m/z* 7769 and *m/z* 8865 were found to be platelet factor 4 (PF4) and beta-thromboglobulin (beta-TG) by tandem mass spectrometry, respectively. The associations of decreased serum PF4 and increased serum beta-TG levels with poor prognosis were confirmed by Western blot. Previous studies suggest that PF4 and beta-TG are involved in the pathogenesis of acute respiratory distress syndrome (ARDS) in a negative and positive way, respectively. Our results suggest that PF4 and beta-TG may also play similar roles in the development of ARDS in SARS patients.

Keywords:
Beta-thromboglobulin / Biomarker / Platelet factor 4 / SELDI ProteinChip technology / Severe acute respiratory syndrome DOI 10.1002/elps.201200002

1 Introduction

Severe acute respiratory syndrome (SARS) is caused by a human SARS coronavirus (SARS-CoV) [1, 2]. The disease affected 32 countries and regions. In the 2003 outbreak, the disease infected more than 8000 people, and killed more than 700 people [3]. Over 30% of the patients required high-flow oxygen therapy; 20–36% required intensive care unit (ICU) admission; and 13–26% developed acute respiratory distress syndrome (ARDS) [4–7]. ARDS-associated causes accounted for about 30% of deaths of the SARS patients in the ICU [8]. The high percentage of patients requiring special treatment/intensive care is a high burden to the medical system. Prognostic indicators/markers are important for risk stratification and guidance of treatment decisions.

SELDI (surface-enhanced laser desorption/ionization) ProteinChip technology is a useful tool in identifying disease-associated proteomic fingerprints in plasma/serum samples [9–12]. Previously, by using a study design avoiding systemic bias, we found that specific proteomic fingerprints, which were composed of 20 proteomic features, were present in the sera of adult SARS patients [13]. The fingerprints not only could differentiate SARS cases from non-SARS cases with similar clinical features, but also separated patients into

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Abbreviations: ARDS, acute respiratory distress syndrome; **beta-TG**, beta-thromboglobulin; **PF4**, platelet factor 4; **SARS**, severe acute respiratory syndrome; **SELDI**, surface-enhanced laser desorption/ionization

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subgroups with different prognoses. These results suggest that some of the SARS-associated proteomic features are potential prognostic markers. In this study, we examined the prognostic values of the individual SARS-associated proteomic features by biostatistical analysis, and deciphered the identities of those with prognostic values. Platelet factor 4 (PF4) and beta-thromboglobulin (beta-TG) were discovered to be potential markers of poor prognosis. These two CXC chemokines may play important roles in the development of ARDS in SARS patients.

2 Materials and methods

2.1 Patient information and proteomic datasets

The patient information and proteomic datasets from our previous study on the serum fingerprints of 39 adult SARS patients were used in this study [13]. The SARS group included 13 males and 26 females and their mean age was 42 years (range 21–88 years); the non-SARS group included 18 males and 21 females and the mean age was 44.4 years (range 20–88 years). The pretreatment serum samples from both SARS and non-SARS groups represented the first time point after hospitalization (3–7 days from onset of fever). All the SARS cases were positive for anti-SARS-CoV IgG antibody serologic test. For the control group, the 39 non-SARS patients were those who had similar symptoms as the SARS patients. They were admitted to the same hospital as the SARS patients, and were later shown to be negative for anti-SARS-CoV antibody serology test after at least 6 weeks of the onset of symptoms.

The serum proteomic profiling data were obtained by using SELDI ProteinChip technology (Ciphergen Biosystems Fremont, CA, USA). Briefly, the serum samples from the diseased and control groups were randomized and blinded to the investigator. CM10 ProteinChip arrays (Ciphergen Biosystems) were used. Two binding conditions were used. One was performed at pH 4.0 while one was performed at pH 9.0. For each binding conditions, samples were analyzed in duplicate. For detailed procedure, please refer to the previous publication [13]. In order to avoid systemic bias, proteomic features were defined as SARS-associated when meeting two criteria: (i) the normalized peak intensities had to be significantly higher/lower in SARS patients, compared to non-SARS subjects; and (ii) the normalized peak intensities had to correlate with two or more clinical/biochemical parameters, indicating their biological meaningfulness. More than 800 common proteomic features were identified and compared between the SARS and non-SARS patient groups. Twenty differential proteomic features were found to be significantly correlated with two or more clinical/biochemical parameters. As a result, there are 20 potential biomarkers for detection of SARS. Fifteen and five had higher and lower levels in the SARS patients, respectively. Hierarchical clustering analysis showed that these 20 biomarkers contained information for identifying the SARS patients at high accuracy (sensitivity = 95%; specificity = 100%), and for separating the SARS patients with subgroups with various prognosis. Patients who required ICU care and/or supplemental oxygen during later treatment were significantly enriched in two clusters ($p = 0.011$). Among the studied SARS patients, the information of supplemental oxygen administration was only available for 38 cases. Therefore, patient information and proteomic datasets from these 38 cases were used in this study.

2.2 Serum samples

The serum samples employed in this study were the same as those used for identification of SARS-associated proteomic features. All samples represented the first-time point after hospitalization. No medications like steroids, ribavirin, traditional Chinese medicine, or intravenous immunoglobulin were given to the patients before sample collection. The samples were stored as aliquots in −70°C until SELDI ProteinChip profiling, protein purification, or Western blot was performed.

2.3 Biostatistic analysis

The data on 20 SARS-associated proteomic features and ten serological variables (ALT, LDH, bilirubin, total protein, albumin, globulin, C-reactive peptide, total white blood cells, lymphocyte count, neutrophil count) from 38 SARS patients before treatment were subjected to forward stepwise multiple logistic regression (SPSS, v18, IBM) for prediction of the ICU admission and/or supplemental oxygen administration in the later period. Forward stepwise multiple logistic regression was a multivariate analysis tool for identifying variables with independent prognostic values. Those variables with completely overlapped prognostic value would give insignificant *p*-values. The normalized peak intensities of the proteomic features were log2 transformed before being subjected to multiple logistic regression.

2.4 Protein purification

The prognostic proteomic features were purified by weak cation-exchange chromatography, as previously described [13]. The functional group of the chromatographic materials, binding, and washing conditions were identical to those used in the SELDI ProteinChip profiling. CM10 ceramic beads (BioSepra, Ciphergen), which has the same weak anionic functional group as the CM10 ProteinChip arrays, was used in the experiment. Briefly, pooled serum samples were first denatured with U9 buffer and diluted with T4 or T9 sampling binding buffers, respectively. After 120-min incubation and subsequent washing, the bound proteins were eluted from the CM10 ceramic beads with 1 M NaCl solution. C18 ZipTips were used to desalt the eluted proteins according to the manufacturer's instructions (Millipore, Bedford, MA, USA). The desalted protein preparations were spotted on the

gold-coated ProteinChip arrays, and examined with the ProteinChip reader to confirm that the purified proteins had the same mass as the targeted SELDI proteomic features. After confirmation, the purified proteins were resolved by 2DE in the absence of reducing chemicals to isolate spots with (i) apparent molecule weights corresponding to the SELDI proteomic features and with (ii) spot intensities showing the same differential patterns in the serum samples collected from the non-SARS patients and the SARS patients with different prognoses, as previously described [12–14]. An immobilized pH gradient (IPG) strip (11cm 3–10 NL, Bio-Rad Laboratories, Hercules, CA, USA) was rehydrated with the sample overnight. For the first dimension IEF separation, the running condition was as follows: 100 V for 10 min, 250 V for 65 min, 500 V for 25 min, 1000 V for 40 min, and finally 8000 V for 140 min. Second dimension SDS-PAGE was performed on 4–12% Bris-Tris polyacrylamide gels (Bio-Rad Laboratories) and the proteins were separated at 200 V for 40 min in ice bath. Proteins on gels were visualized either by colloidal blue (Invitrogen, San Diego, CA, USA) or silver staining (GE Healthcare, Piscataway, NJ, USA). The gel images were then digitalized with a densitometer and were analyzed by the PDQuest gel analysis software (version 7.3.0, Bio-Rad). Protein spots in the 2D-gel with apparent molecular weights matched with the prognostic proteomic features were excised and subjected to mass spectrometry (MS) analysis.

2.5 Protein identification by MS

The identity of the protein in a gel spot was obtained by tandem mass spectrometer, as previously described [12, 13]. Briefly, the gel pieces were destained, reduced with 1.75% DTT, alkylated with 350 mM iodoacetamide (IAA), and digested overnight with modified porcine trypsin (sequencing grade, Promega, Madison, WI). The tryptic digest was harvested and cleaned up with C18 ZipTips (Millipore). The cleaned tryptic peptides were subjected to tandem MS analysis using the ABI 4700 MALDI-TOF/TOF system (Applied Biosystems, Carlsbad, CA, USA) with α-cyano 4-hydroxy cinnamic acid as matrix. The MS/MS spectra were then processed with Data Explorer software (Applied Biosystems, version 4.4). The spectra were subjected to Gaussian smoothing with filter width of 5 points, and the baselines were corrected with default settings. Peaks were detected using a signal to noise threshold of 15. The fragment masses and intensities of each MS/MS mass spectrum were subjected to online Mascot MS/MS ion search (http://www.matrixscience.com/) to obtain the protein identities. For the search parameters, one missed cleavage in trypsin digestion was allowed; partial oxidation of methionine, phosphorylation of serine/threonine/tyrosine, and iodoacetamide modification of cysteine residues were selected. The error tolerance values of the parent peptides and the MS/MS ion masses were 0.1 and 0.3 Da, respectively. A protein identification result was considered significant when the MS/MS ion profile matched a known protein in the NCBInr database with a p-value <0.05. For each identified protein, an accession number in the UniProt protein database was reported when available.

2.6 Western blot

For ensuring unbiased comparison among the patient samples, fixed volumes of individual serum samples were loaded into a SDS-gel. Furthermore, samples for comparison (e.g. SARS cases versus non-SARS cases) were separated in the same SDS-gel, transferred to the same membrane and subjected to immunoblotting in a single experiment. Serum samples (2 μL) were first denatured by addition of 4 uL of U9 buffer (9 mol/L urea, 20 g/L CHAPS, 50 mmol/L Tris-HCl, pH 9.0) and incubated on ice for 20 min. Two microliters of the U9 denatured serum samples (equivalent to 0.67 μL of neat serum) were denatured again and reduced by boiling in reducing SDS sample loading buffer, and separated with precast SDS-polyacrylamide gels (Bio-Rad). The samples were then transferred to PVDF membranes by electroblotting (Bio-Rad). The membranes were blocked with BSA and incubated overnight at 4°C with 0.2 μg/mL rabbit polyclonal anti-human platelet factor-4 (PF-4) antibody (Abcam, UK) or 0.2 μg/mL rabbit polyclonal anti-human beta-TG antibody (also called anti-human NAP2 antibody, Abcam, UK). Afterwards, the membranes were washed and incubated with a horseradish peroxidase conjugated goat anti-rabbit polyclonal antibody (Dako, Denmark) at 1:2500 dilution. Detection was performed using the ECL Plus Western Blotting Detection solution (GE Healthcare) and the signal was visualized with chemiluminescence film (GE Healthcare). The results were finally digitalized with the densitometer and analyzed with the Quantity One gel analysis software (version 4.5.1; Bio-Rad).

3 Results

3.1 Prognostic proteomic features

Among the 20 SARS-associated proteomic features and ten serological variables (ALT, LDH, bilirubin, total protein, albumin, globulin, C-reactive peptide, total white blood cells, lymphocyte count, neutrophil count), multiple logistic regress analyses identified four proteomic features (m/z 6634, m/z 7769, m/z 8635, m/z 8865) as statistically significant prognostic markers for supplemental oxygen administration or ICU admission in the later period. However, none of the serological variables was statistically significant. Peak intensities of the m/z 6634 and m/z 7769 proteomic features were negatively associated with supplemental oxygen administration, whereas peak intensities of m/z 8865 and m/z 8635 proteomic features was positively associated with supplemental oxygen administration and ICU admission, respectively. Table 1 summarizes the odds ratios and the p-values of these prognostic proteomic features. Figure 1 shows the gel views of the representative SELDI mass spectra of two prognostic

Table 1. Summary of the SARS-associated proteomic features identified as prognostic markers for supplemental oxygen administration or ICU admission

m/z, mean (minimum to maximum)	Direction of prediction	Adverse outcomes	Odds ratio (95% confidence interval)	*p*-value
6634 (6626–6643)	Negative	Supplemental oxygen administration	0.09 (0.02–0.50)	0.006
7769 (7761–7776)	Negative	Supplemental oxygen administration	0.26 (0.09–0.82)	0.021
8635 (8630–8641)	Positive	ICU admission	4.02 (1.20–13.44)	0.024
8865 (8856–8874)	Positive	Supplemental oxygen administration	5.10 (1.06–24.5)	0.042

The odds ratios for doubling of normalized peak intensities and *p*-values were determined by multiple logistic regression (forward stepwise).

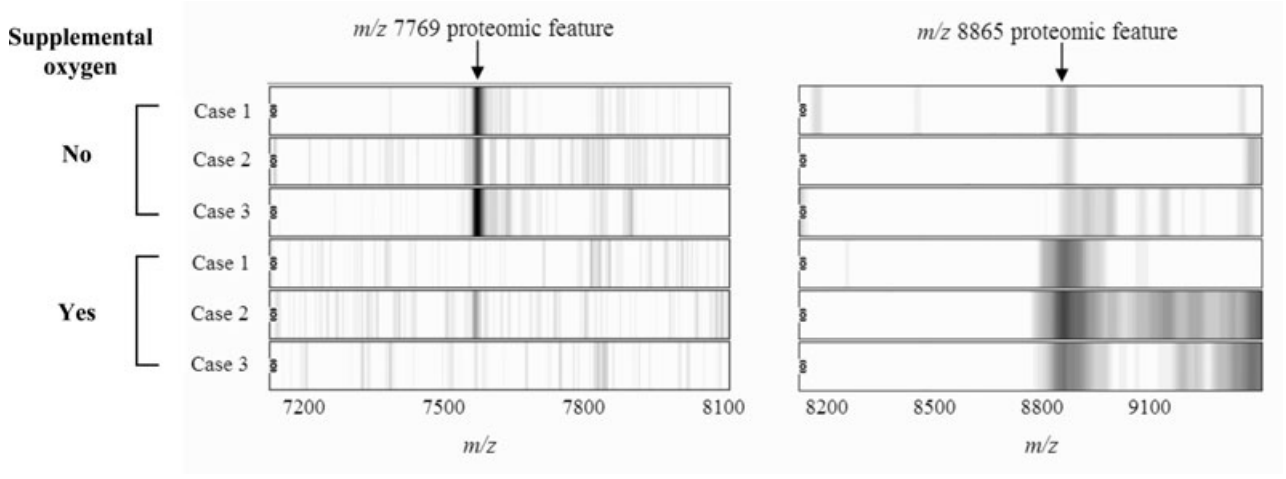


Figure 1. Representative gel views of the SELDI ProteinChip mass spectra from serum samples of the SARS cases with favorable and adverse outcomes. The proteomic feature of *m/z* 7769 was negatively associated with supplemental oxygen administration, whereas the proteomic feature of *m/z* 8865 was positively associated with supplemental oxygen administration.

proteomic features in SARS patients with and without adverse outcome.

3.2 Protein identities of proteomic feature *m/z* 7769 and *m/z* 8865

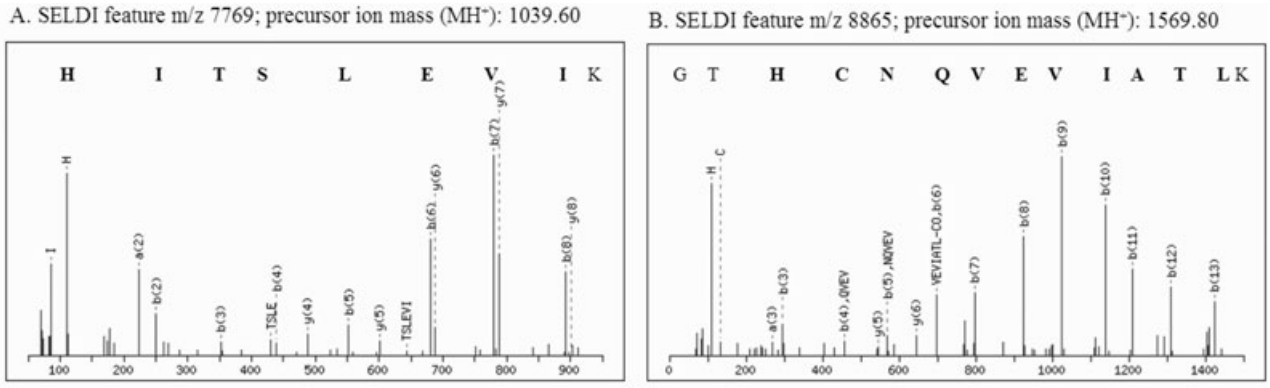
Attempts were made to purify and identify the four prognostic proteomic features (Supporting Information Fig. S1). By tandem MS, we successfully uncovered the identities of the protein spots having apparent molecular weight corresponding to *m/z* 7769 and *m/z* 8865, which were platelet factor-4 (PF4) and beta-TG, respectively (Fig. 2). The raw mass spectrometric data are provided in the Supporting Information data files.

3.3 Validation of differential patterns of PF4 and beta-TG by Western blot

To confirm the differential patterns and the identities of the *m/z* 7769 and *m/z* 8865 proteomic features, serum samples were subjected to Western blot analysis using specific antibodies against PF4 and beta-TG. The beta-TG antibody also detected the precursor protein of beta-TG, which was connective tissue-activating peptide III (CTAPIII) and appeared as a weak upper band in the Western blot (Fig. 3). In agreement with the proteomic profiling data, serum PF4 levels and beta-TG levels in the SARS patients were significantly lower and higher than those in non-SARS patients, respectively (Fig. 3A, Supporting Information Fig. S2A and B). When comparing between the SARS patients with or without supplemental oxygen administration in the later period, serum PF4 levels and beta-TG levels were significantly lower and higher in the patients with supplemental oxygen administration, respectively (Fig. 3B, Supporting Information Fig. S2C and D).

4 Discussion

The emergency of SARS in 2003 has stimulated research on innate immune response to coronaviruses. Because SARS has not recurred since its first outbreak, most of the research studies on pathogenesis of SARS have been based on the use of animal or cell line models [15]. This is one of the major reasons leading to our limited knowledge on SARS pathogenesis. Coronavirus can cause inflammation and damage in the lung. In severe cases, ARDS occurs during the acute phase of SARS coronavirus (SARS-CoV) infection, whereas diffuse alveolar damage occurs during the organizing phase. Both are considered as clinically devastating end-stage lung diseases. Various studies have indicated that the SARS-CoV can escape



C. Summary of MS/MS matching

SELDI feature (m/z)	Protein Identity (theoretical m/z)	Mowse Score (cut-off value when P<0.05)	UniProt entry	Parent ion mass (MH^+), mass deviation	Corresponding positions	MS/MS peptides Sequence	Mowse Score (individual peptide)
7769	Platelet factor 4	100 (47)	P02776	1039.60, -0.01	23-31	R.HITSLEVIK.A	63
	(7775)			1461.80, -0.01	50-61	R.KICLDLQAPLYK.K + (C) [a]	38
8865	beta-thromboglobulin	231 (47)	P02775	1056.51, 0.00	57-65	K.ICLDPDAPR.I + (C)	38
	(8868)			1569.80, -0.01	39-52	K.GTHCNQVEVIATLK.D + (C)	98
				1724.84, 0.00	1-15	K.GKEESLDSDLYAELR.C	97

[a] (c) = carbamidomethylation

Figure 2. MS/MS identification of two prognostic proteomic features of *m/z* 7769 and *m/z* 8865. (A) and (B) MS/MS spectra of one of the tryptic peptides from the *m/z* 7769 and *m/z* 8865 proteomic features, respectively. Positions of the amino acids are indicated according to the b-series ions. (C) Summary of the MS/MS matching results, the Mowse score, and the required score for significant matching (p <0.05) are shown.

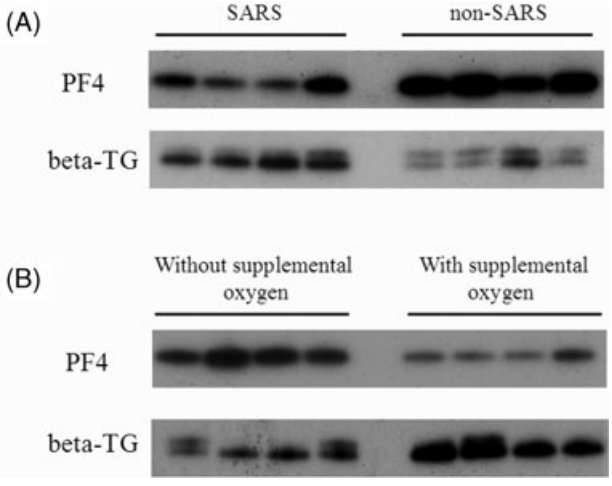


Figure 3. Western blotting of PF4 and beta-TG in serum samples of representative cases. (A) Serum levels of PF4 and beta-TG in SARS and non-SARS patients, respectively. (B) Serum levels in SARS patients with and without supplemental oxygen administration in the later period. The beta-TG antibody also recognized the precursor protein of beta-TG, which appeared as a weak upper band in the Western blot. The corresponding densitometry data are provided in Supporting Information Fig. S2.

from induction of the antiviral type I interferons in tissue cells, while chemokines IP-10 or IL-8 are strongly upregulated in the infected cells [16]. To the best of our knowledge, this is the first study demonstrating the prognostic values of serum PF4 and beta-TG in predicting the need of supplemental oxygen in the SARS patients. As both PF4 and beta-TG are CXC chemokines involved in the modulation of immune response, the results of this study also provide novel insights on the pathogenesis of SARS.

PF4, also called CXCL4, is produced and secreted by various cell types, although previously it was believed that it was exclusively produced by platelets and megakaryocytes [17]. Biological functions of PF4 have not been fully understood. It has been shown to participate in various functions in the immune system, such as promoting interaction between neutrophils and endothelial cells [18], inhibiting T-cell proliferation [19], etc. Beta-TG was the first protein of the beta-TG family to be described [20]. Members of beta-TG family are CXC chemokines, comprising PBP, CTAP-III, beta-TG, and NAP-2. They are encoded by the same gene PPBP with variations in the number of N-terminal amino acids [20]. CTAP-III is considered as the precursor of beta-TG and NAP-2. The function roles of beta-TG have not been completely elucidated.

Evidence has shown that members of the beta-TG family have different abilities in neutrophil chemoattraction, stimulation of neutrophil adhesion, and transendothelial migration of neutrophils [21]. NAP-2, also called CXCL7, is the key and most potent member responsible for recruitment and activation of neutrophils [22]. Although both NAP-2 and IL-8 play important roles in neutrophil activation during inflammatory reactions, they act through different molecular pathways [23]. The functional relationship among IL-8, PF4, and beta-TG is highly complex. On one hand, PF4 and beta-TG family members have divergent roles in neutrophil regulation [20]. PF4 inhibits production of NAP-2 from its precursor CTAP-III in both stimulated and unstimulated neutrophils and mast cells [24]. On the other hand, PF4 induces human natural killer cells to synthesize and release IL-8 [25]. Between 13% and 26% of the SARS patients developed ARDS [4–7]. ARDS is regarded as the severe stage of acute lung injury. Inflammatory mediators play an important role in the pathogenesis of ARDS through recruitment and activation of neutrophils [26, 27]. High levels of beta-TG and NAP-2 were found in the bronchoalveolar lavage fluid of patients with ARDS [28, 29]. Lower level of PF4 was also observed in the bronchoalveolar lavage fluid of ARDS patients, although this difference was not statistically significant [28]. In view of the findings from the previous and current studies, we here postulate that increased beta-TG and decreased PF4 levels may play important roles in the development of ARDS in the SARS patients.

PF4 and beta-TG have been repeatedly reported to be biomarkers for various pathologies and conditions. An increased PF4 level in plasma is usually associated with an increase of beta-TF. Conventionally, elevated plasma levels of plasma PF4 and beta-TG are used as indicators of platelet activation. Increased plasma levels of both PF4 and beta-TG have been proposed to be used as diagnostic/prognostic biomarkers for various kinds of diseases, such as diagnosis of megakaryoblastic leukemia [30], prediction of thrombotic complications in patients with artificial heart valves [31], prognosis of cerebral infarction [32, 33], etc. Moreover, it is not uncommon that plasma/serum proteomic profiling identified an alternated plasma/serum level of only either PF4 or beta-TF level, but not both, as a potential diagnostic/prognostic biomarker. For example, elevation of PF4 was associated with poor response to infliximab in rheumatoid arthritis [34]. Decreased PF4 level was associated with acute lymphoblastic leukemia [35], whereas decreased beta-TG level was observed in patients with pancreatic cancer [36]. A parallel observation of decreased PF4 level and increased beta-TG level in blood has not been reported until the present study on SARS. In ARDS, similar dysregulation pattern was observed in the bronchoalveolar lavage fluid [28, 29]. Therefore, it is very likely that a combination of decreased PF4 level and increased beta-TG level in blood is a specific indicator for poor prognosis in SARS.

In conclusion, four proteomic features were found to be potential prognostic markers of SARS by biostatistical analysis. Two of them were identified as CXC chemokines, PF4, and beta-TG. Decreased PF4 and increased beta-TG in the pretreatment serum samples were associated with the need of supplemental oxygen in the later treatment period. Previous studies suggest that PF4 and beta-TG may be involved in the pathogenesis of ARDS in a negative and positive way, respectively. Our results, hence, suggest that PF4 and beta-TG may also play important roles in the development of ARDS in SARS patients.

The work was supported by the Research Fund for the Control of Infectious Diseases (RFCID) from the Health, Welfare, and Food Bureau of the Hong Kong SAR Government and by the Li Ka Shing Foundation.

The authors have declared no conflict of interest.

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