

The use of proteomics in the discovery of serum biomarkers from patients with severe acute respiratory syndrome

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Severe acute respiratory syndrome (SARS) is a new infectious disease with a global impact. Understanding its pathogenesis and developing specific diagnostic methods for its early diagnosis are crucial for the effective management and control of this disease. By using proteomic technology, truncated forms of α_1 -antitrypsin (TF- α_1 -AT) were found to increase significantly and consistently in sera of SARS patients compared to control subjects. The result showed a sensitivity of 100% for SARS patients and a specificity of 92.8% for controls. Furthermore, the levels of these proteins significantly correlated with certain clinico-pathological parameters. The dramatic increase in TF- α_1 -AT may be the result of degradation of α_1 -AT. As α_1 -AT plays an important role in the protection of lung function, its degradation may be an important factor in the pathogenesis of SARS. These findings indicate that increased TF- α_1 -AT may be therapeutically relevant, and may also be a useful biological marker for the diagnosis of SARS.

Keywords: α_1 -antitrypsin / Biomarker / Complement 4 / Serum amyloid A / Severe acute respiratory syndrome

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1 Introduction

Severe acute respiratory syndrome (SARS), a recently-emergent infectious disease, has been responsible for 916 deaths worldwide out of 8422 infected cases (as of August 7, 2003). A novel coronavirus (SCV) has been identified as the aetiological agent [1–3]. Although the spread of this devastating disease appears for the

moment to have been contained, further serious outbreaks of SARS cannot be ruled out. Efforts should therefore be made to find alternative diagnostic methods, therapeutics and vaccines. At present, confirmation of suspected clinical diagnosis is exclusively based on virus isolation, RT-PCR and serology. These methods still lack sensitivity for the diagnosis of SARS in the first few days of the illness [4] as the virus load in the upper respiratory tract is low during the first week of illness and peaks around day 10 of illness [5]. Detection of antibodies to SCV using immunofluorescence and well-validated ELISA tests has been a very reliable means for confirming the

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Abbreviations: α_1 -AT, α_1 -antitrypsin; C4, complement 4; SAA, serum amyloid A; SARS, severe acute respiratory syndrome; SCV, coronavirus; TF- α_1 -AT, truncated form of α_1 -antitrypsin

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diagnosis. However, the antibody response appears only around day 10 of the illness or even later [5]. Early detection of the disease is crucial for effective treatment and for the early isolation of patients. A quantitative real-time RT-PCR assay has been developed to detect SCV. Its sensitivity reached 80% in the first 3 days of illness, a higher level than the conventional RT-PCR assay [6]. Furthermore, evolving methods for analysis of the expression of protein are opening new opportunities of disease progression and providing insights into disease mechanisms.

Proteomic technology is a recent development in genomics, which couples the techniques of protein analysis with bioinformatics and has impressive potential in biomedical studies. Proteomic analysis offers a powerful approach to identify disease-associated proteins that can be used as biomarkers for diagnosis and as drug targets for treatment [7, 8]. Currently, 2-DE MS and bioinformatics are the main tools for biosample analysis and protein identification. Proteomics can also be an important tool for identifying virulence determinants, antigens, and vaccine candidates, since changes in the proteome depend on developmental stages, disease states, or environmental conditions. The identification of SARS-associated proteins by means of proteomic analysis may provide significant information for the elucidation of virulence factors, antigens and vaccines. These are important for diagnosis, therapy and protection from the disease. In this study, we used proteomics to detect serum biomarkers in SARS. We found that at least three clusters of protein were significantly changed in serum samples from patients with SARS compared with those in normal and SCV negative serum samples. The alterations of these proteins were evident not only in quantity, but also in the patterns (or specificity) and correlated with clinico-pathological parameters. These proteins could be useful serum biomarkers for SCV diagnosis, and might also offer encouraging targets for therapy.

2 Methods

2.1 Human subjects

Serum samples from the Department of Clinic Microbiology, Queen Mary Hospital, The University of Hong Kong, were used in this study. The samples were taken from thirteen patients suffering from an illness that fit the World Health Organization definition of SARS. These patients were virologically confirmed. RT-PCR was used to detect SCV RNA in the nasopharyngeal aspirate, endotracheal aspirates, sputum, urine and faecal samples for all clinical samples. The convalescent sera were tested for SCV IgG. Blood samples were collected from (i) SARS patients on the day of admission (4.9 ± 3.9 days

after onset of illness, $n = 13$); (ii) the same patients after one week of treatment with corticosteroids and ribavirin ($n = 13$); (iii) patients with pneumonia caused by non SCV such as bacteria, adenovirus and influenza viruses ($n = 12$; age- and sex-matched) as disease controls; and (iv) healthy individuals as normal controls ($n = 14$). Consent was obtained according to the regulations of the Human Tissue Committee, Queen Mary Hospital, The University of Hong Kong. The study was approved by the local ethics committee. All serum samples were stored at -80°C until use. The protein concentration of all samples was determined by the Bradford method.

2.2 2-DE

2-DE was carried out with Amersham Biosystems IPG-phor IEF and Ettan Dalt six electrophoresis units (Amersham Biosciences, Uppsala, Sweden) in accordance with a protocol described previously [9]. Serum samples with protein amounts of 250 μg were applied to the 2-DE and run in groups side by side. Electrophoresis was performed in duplicates at least for each sample to ensure reproducibility. All gels were visualized by Silver-staining.

2.3 Image analysis and MS peptide fingerprint sequencing

Image acquisition and analysis were performed by using an ImageScanner (Amersham, Biosciences) and the ImageMaster 2D Elite software (Amersham, Biosciences) [10]. A comparison was made among gel images of normal, SARS, treated SARS and symptom control samples in groups. Normalized volume differences were statistically calculated for all the cases. Consistently and significantly different spots were selected for analysis with MALDI-TOF MS. Protein spots were cut out in small pieces and subjected to in-gel tryptic digestion overnight. Peptide mass spectra were recorded and parameters for spectra acquisition were used as stated previously. Protein identification was performed by searching in NCBI protein database using MS-FIT (<http://prospector.ucsf.edu/>).

2.4 2-D Western blotting

Serum samples were run on 2-D gels using the same conditions used for 2-DE (see section 2.2). The separated proteins in the corresponding areas of gels were transferred onto polyvinylidene fluoride membranes and incubated overnight at 4°C with a blocking buffer containing TBST (Tris-buffered saline, 0.1% Tween 20) and 5% skimmed milk. Membranes were washed with TBST and incubated with antihuman serum amyloid A (SAA) sheep

polyclonal antibody at a 1:1000 dilution (Calbiochem, CA, USA) or anti α_1 -antitrypsin (α_1 -AT) rabbit polyclonal antibody at a 1:500 dilution (Neo Markers, CA, USA) for 1 h at room temperature or overnight at 4°C. After a further washing with TBST, the membranes were blotted with a secondary antibody conjugated with horseradish peroxidase at a 1:10 000 dilution for 1 h, and then detected with enhanced chemiluminescence (Pierce, Rockford, IL, USA) for 1 min.

2.5 Immunonephelometry

α_1 -AT in human serum was measured by immunonephelometry using Dade Behring ProSpec [11]. α_1 -AT reacts with rabbit antihuman α_1 -AT antiserum to form immune complexes which cause light scattering in the nephelometer. The amount of light scatter is directly proportional to the α_1 -AT concentration, which can be determined from a stored standard curve.

2.6 Statistical analysis

To evaluate the significance of correlation between serum levels of tested proteins and clinico-pathological parameters, the Fisher transformation of the Pearson correlation was employed to compute *p*-values. The best linear combinations with weight ranging between 0 and 1 of the standardized serum levels of the tested proteins were obtained, with the aim of identifying the combination of proteins that best described the correlations. *p* values of < 0.05 were considered to be statistically significant.

3 Results

3.1 Diagnostic groupings

The mean age of the 13 patients with SARS (nine men and four women) was 41.5 ± 9.5 years (median 45; range from 25 to 56). All patients were otherwise in good health. The results of the haematological and biochemical investiga-

tions are shown in Table 1, together with clinical characteristics after one week of administration of corticosteroids and ribavirin. There was a resolution of fever and improvement in heart rate and oxygen saturation after one week of treatment (data not shown). There was also an increasing trend toward improved leucocyte and platelet counts (Table 1). These data showed that the one-week treatment was effective.

Table 1. Clinical laboratory profiles of SARS patients in this study

	Before treatment mean \pm SD, <i>n</i> = 13	After treatment mean \pm SD, <i>n</i> = 13
Age (years)	41.5 \pm 9.5	41.5 \pm 9.5
Male/female ratio	9/4	9/4
Haemoglobin (Hb, g/dL)	13.7 \pm 1.4	13.1 \pm 1.5
Leukocyte count ($\times 10^9$ /L)	6.8 \pm 4.1	10.5 \pm 3.8
Neutrophil count ($\times 10^9$ /L)	5.5 \pm 3.9	9.2 \pm 3.7
Lymphocyte count ($\times 10^9$ /L)	0.8 \pm 0.3	0.75 \pm 0.6
Monocyte count ($\times 10^9$ /L)	0.5 \pm 0.3	0.46 \pm 0.35
Platelet count ($\times 10^9$ /L)	212.5 \pm 102.4	375.6 \pm 147.9
CRP	6.2 \pm 4.8	< 0.32
ALT (U/L)	74.5 \pm 55.6	115.6 \pm 79.4
Creatinine (μ M/L)	93.4 \pm 22.4	80.7 \pm 24.7
Na	135.4 \pm 5.1	138.0 \pm 2.6
K	3.8 \pm 0.4	4.1 \pm 0.6
Cl	101.4 \pm 4.01	99.8 \pm 4.4

3.2 Protein profiles of SARS

When 250 μ g of serum samples were applied to the 2-DE separation, well-separated protein patterns were obtained and were found to be similar to the plasma pattern in the SWISS-2D database (<http://www.expasy.ch/ch2d/>), except that they lacked fibrinogen. Gel image comparisons of the four groups of sera revealed that at least three clusters of protein spots were consistently and significantly altered in the serum from patients with SARS. Spot volume comparison was made between four groups of samples using the ImageMaster program. Table 2

Table 2. Summary of protein alterations

Protein ID	Experimental <i>M_r</i> / <i>pI</i>	Normal (<i>n</i> = 14) (% vol)	SARS (<i>n</i> = 13) (% vol)	Treated SARS (<i>n</i> = 13) (%vol)	NonSARS pneumonia (<i>n</i> = 12) (% vol)
α_1 -AT	47 kD / 5.1	0.057 \pm 0.063	0.260 \pm 0.091	0.106 \pm 0.065	0.035 \pm 0.027
C4 fragment	30 kD / 6.7	0.097 \pm 0.077	0.419 \pm 0.099	0.146 \pm 0.083	0.185 \pm 0.038
SAA	12 kD / 5.5–6.8	0.018 \pm 0.023	0.640 \pm 0.273	1.240 \pm 0.366	0.645 \pm 0.485

Table 3. Results of MALDI-TOF mass spectra and database searching for protein identification

Protein	Peptides matched	Sequence coverage	Confirmation method
TF- α_1 -AT	9–11	22–29%	Western blot
Complement C4 fragments	10–13	4–6%	Plasma map ^{a)}
Serum amyloid A	5–11	54–68%	Western blot

a) SWISS-PROT 2D database (<http://www.expasy.ch/ch2d/>)

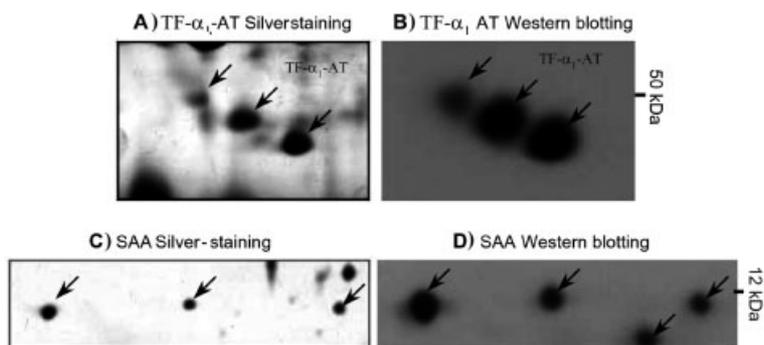


Figure 1. Patterns of truncated α_1 -AT (TF- α_1 -AT) and SAA in SARS patients detected by 2-D gel (A and C) and confirmed by Western blotting (B and D).

summarizes the experimental locations of the altered protein spots and the statistical data for these spots (or the sum of spot clusters) with normalized volumes (% vol). Overall, the spot volumes of these three protein groups were significantly elevated from very low or undetectable levels in the sera from healthy subjects to much higher levels in sera from SARS patients. The altered spots were excised from gels and subjected to mass spectrometric analysis and database searching. The results showed that the protein spots belong to α_1 -AT, complement C4 protein (C4) and SAA respectively. The peptide fingerprinting and database matching results are summarized in Table 3. As the spots of α_1 -AT and C4 appeared in the gel regions with M_r lower than their full-length molecules, they are evidently truncated or cleaved protein fragments. This conclusion is also consistent with the finding of peptide sequences resulting from database matching: the matched peptides do not cover the *N*-terminus of the protein α_1 -AT, and only the last ~5% of the C4 sequence (Table 3).

The identity of protein C4 was also confirmed by comparing its spot pattern and M_r/pI region to the plasma gel in SWISS-2D proteome (<http://www.expasy.ch/ch2d/>). Proteins α_1 -AT and SAA are not shown in the normal plasma database. Western blotting was therefore performed to validate the identification. Figure 1 shows the 2-D Silver-staining and Western blotting side by side, indicating the specific immunochemical interaction between both α_1 -AT and SAA proteins and their antibodies in

the corresponding areas of the 2-D gels. Western blotting also detected another SAA isoform in the lower M_r area that was not detectable in the corresponding Silver-stained gel (Fig. 1D). Figure 2 demonstrates the protein alterations in the AT area. The *N*-terminal truncated α_1 -AT has three peptides or isoforms (Fig. 2B). The serum levels of these isoforms are very low or undetectable in the sera from healthy and patients with nonSARS pneumonia (Fig. 2A and C). However, an approximately 4.5-fold increase of the truncated α_1 -AT level was observed in

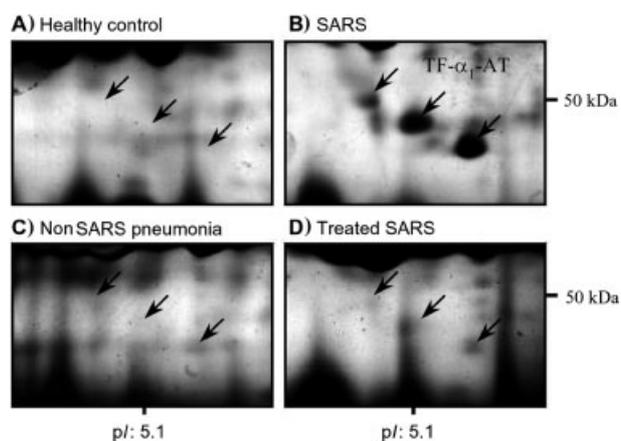


Figure 2. Patterns of TF- α_1 -AT in serum from healthy controls, patients with SARS before or after treatment, and nonSARS disease controls.

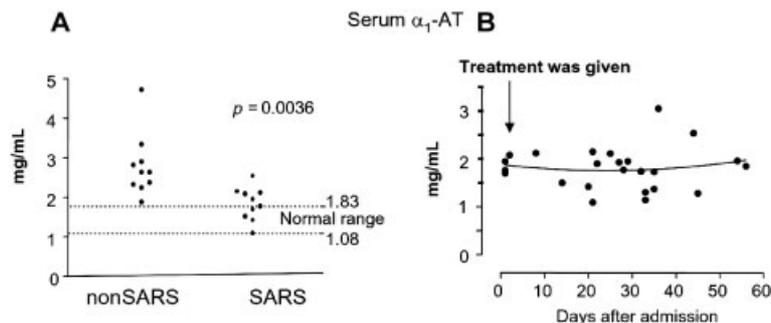


Figure 3. (A) Total serum α_1 -AT concentrations (including complete and truncated forms) from patients with SARS and nonSARS disease controls in early stage. (B) Total serum α_1 -AT concentrations from patients with SARS plotted against time since admission ($n = 23$).

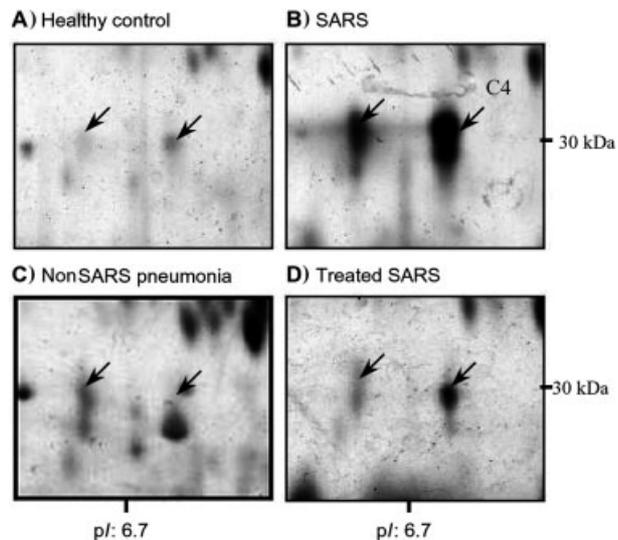


Figure 4. Patterns of C4 in serum from healthy controls, patients with SARS before or after treatment, and nonSARS disease controls.

SARS patients (Fig. 2B), and this level was considerably decreased in the sera from SARS patients after treatment for one week (Fig. 2D, Table 2).

Our results showed not only that α_1 -AT was truncated in SARS patients, but that the serum α_1 -AT concentration (including complete and truncated forms) was also significantly lower than in patients with nonSARS pneumonia (Fig. 3A). However, there were no differences in serum α_1 -AT concentrations between the early and late stages (Fig. 3B). A similar trend of alteration was found in the area of the C4 peptides (Fig. 4). The serum concentration of two C4 fragments was dramatically elevated in SARS patients compared to healthy and nonSARS pneumonia subjects, but then significantly decreased in the same patients after treatment (Table 2). The pattern of protein change in the SAA area was different (Fig. 5). Three isoforms of full-length SAA could be identified at a very low level in healthy subjects (Fig. 5A). However, serum SAA was significantly increased in SARS patients, and was

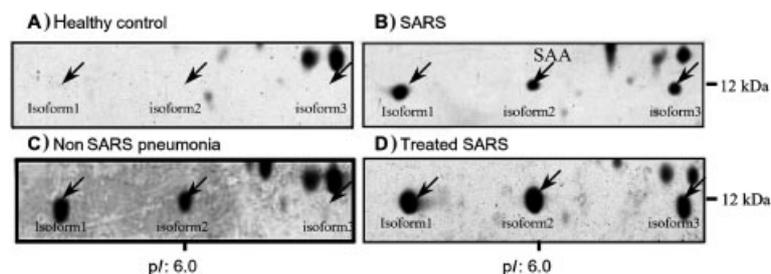


Figure 5. Patterns of SAA in serum from healthy controls, patients with SARS before or after treatment, and nonSARS disease controls.

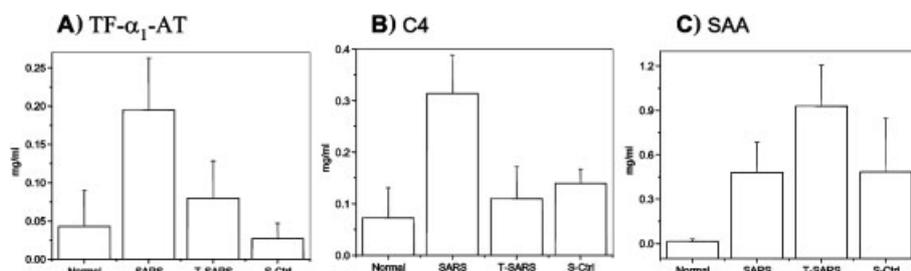


Figure 6. Protein alterations in serum samples of healthy controls (normal), SARS patients before (SARS) or after treatment (T-SARS), and nonSARS disease controls (S-Ctrl).

further elevated after one week of treatment. Meanwhile, a similar concentration of SAA was also observed in the sera of nonSARS pneumonia subjects (Table 2). Furthermore, isoform 3 (Fig. 5) was not detectable in the sera from healthy controls and patients with nonSARS pneumonia, suggesting it may be associated solely with the SARS patients. Figure 6 summarizes the plots for the alterations of the three protein levels in the four groups of subjects (healthy controls, SARS patients, treated SARS patients, and nonSARS disease controls).

3.3 The relationship between the levels of TF- α_1 -AT, C4, SAA and clinicopathologic parameters

Overall, in patients with SARS, the level of TF- α_1 -AT was significantly correlated with lymphocyte count ($r = 0.52$, $p = 0.04$) and CPK levels ($r = 0.95$, $p = 0.0$) (Table 4). SAA level correlated with haemoglobin concentration ($r = 0.57$, $p = 0.02$), monocyte count ($r = 0.68$, $p = 0.0$) and ALT ($r = 0.56$, $p = 0.02$) (Table 4). However, C4 levels did not correlate with any clinicopathologic parameters (data not shown).

3.4 Combined evaluation

To obtain a better correlation between biomarkers and clinico-pathological features, TF- α_1 -AT was combined with C4, and TF- α_1 -AT with SAA, to analyze their additive effect on

Table 4. Correlation between markers and clinico-pathological features

	TF- α_1 -AT		SAA	
	r	p value	r	p value
Age (years)	-0.37	0.16	0.38	0.15
Haemoglobin (Hb, g/dL)	0.04	0.90	0.57	0.02*
Leukocyte count ($\times 10^9/L$)	-0.17	0.55	0.32	0.24
Neutrophil count ($\times 10^9/L$)	-0.19	0.48	0.26	0.33
Lymphocyte count ($\times 10^9/L$)	0.52	0.04*	0.25	0.35
Monocyte count ($\times 10^9/L$)	-0.37	0.17	0.68	0.00*
Platelet count ($\times 10^9/L$)	-0.10	0.72	0.14	0.61
CRP	0.16	0.56	0.46	0.07
ALT (U/L)	-0.12	0.67	0.56	0.02*
Creatinine ($\mu M/L$)	0.02	0.94	0.25	0.35
CPK	0.95	0.00*	0.30	0.26
Na	-0.15	0.59	-0.09	0.74
K	-0.41	0.12	-0.07	0.81
Cl	-0.41	0.12	-0.10	0.73

* Significant correlations, see Section 3.3

the correlation with clinico-pathological features in SARS patients (Table 5). These combinations showed stronger significant correlations with the corresponding clinical information. Figure 7 shows the correlations between clinico-pathological characteristics and protein combinations.

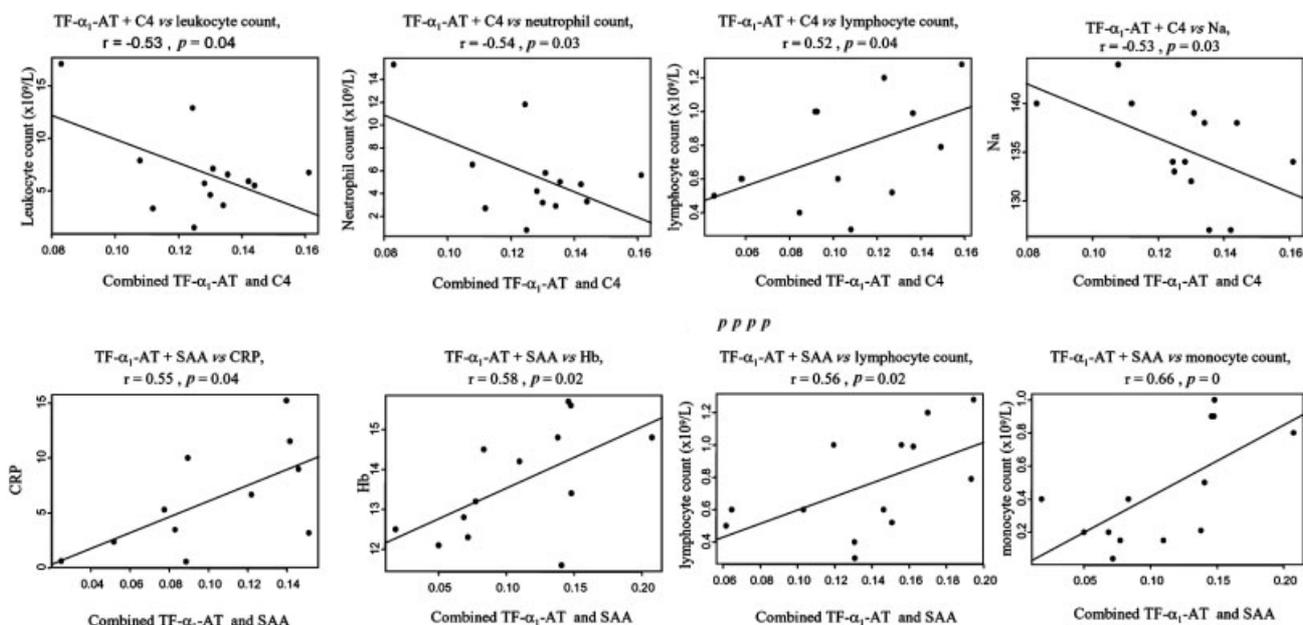


Figure 7. Correlation between combined markers and clinico-pathological features. The best linear combinations with weights ranging between 0 and 1 of the standardized serum levels of the tested proteins was obtained, with the aim of identifying the combination of proteins that best described the correlations. P values of < 0.05 were considered to be statistically significant.

4 Discussion

We used high resolution 2-D gel and image analysis to examine archived serum samples from patients with SARS and controls. To our knowledge, our study is the first report on the serum proteomes of SARS. TF- α_1 -AT, C4 and SAA were identified as being consistently over-expressed in all 13 patients, and reached levels 4.6, 4.3 and 34.3 times higher than in healthy subjects. The frequency of elevated levels of these proteins in SARS was 100% in all 13 patients examined. TF- α_1 -AT was the most specific (specificity of 92.8% for all controls). It was absent in patients with nonSARS pneumonia, and although it was positive in two of the 14 healthy controls, the levels were much lower than in the SARS patients (data not shown). According to our data, most patients showed a clinical improvement after one week of treatment. Interestingly, serum TF- α_1 -AT levels declined in SARS patients after one week of treatment with corticosteroids and ribavirin, suggesting either that α_1 -AT may be truncated transiently in the early stage of SARS, or that the treatment might be effective. A correlation was found between high levels of these proteins and specific clinicopathological parameters. Furthermore, a weighted combined analysis showed stronger correlations (Table 5). The strong correlations between the levels of these proteins and CRP, Hb, lymphocyte count, and monocyte count indicate that these proteins may be involved in the development of the disease, and may serve as useful markers for monitoring disease progression in SARS patients. Therefore, targeting these proteins offers the potential for controlling the progression of SARS.

Proteases are involved in a wide variety of biological processes, including inflammation and tissue injury. The activity of proteases from dying cells such as macrophages

and neutrophils is controlled by the inhibitory actions of antiproteases such as α_1 -AT [12]. Therefore, adequate activity of this inhibitor is critical for maintenance of protease-antiprotease homeostasis and prevention against proteolytic tissue damage [13, 14]. α_1 -AT is a protease inhibitor found in bronchoalveolar lavage fluid from the human lung [15]. It was found to reduce lung injury and fibrosis. This acute phase protein is produced by normal bronchial epithelial cells and macrophages stimulated with infectious particles. Furthermore, protease released from inflammatory cells such as macrophages, the predominant cells in SARS [16], may contribute to the lung fibrosis which is a common sequela in many SARS patients. Protease-antiprotease imbalances are recognized in several inflammatory lung diseases, including adult respiratory distress syndrome (ARDS) [16]. About 20% of SARS patients developed ARDS [17].

Our current data showed that the serum α_1 -AT level in SARS patients was lower than in patients with nonSARS pneumonia, suggesting that the capacity of self protection is impaired in SARS patients in response to infection with SCV. In contrast, patients with nonSARS pneumonia seem to be able to produce more α_1 -AT (total concentration including complete and truncated forms), thereby limiting or reducing lung damage. Furthermore, the increased level of truncated α_1 -AT may be the result of the proteolytic cleavage of functional α_1 -AT due to an unknown mechanism of SCV infection, so that the activity of α_1 -AT may be reduced by truncation. It is therefore hypothesized that the combination of a lower concentration and lower activity of α_1 -AT in SARS patients is probably associated with lung failure and contributes to the development of ARDS. However, this hypothesis requires further investigation. In addition, our results showed that one-week treatment significantly correlated with the level

Table 5. Correlation between combined markers and clinico-pathological features

	TF- α_1 -AT and C4			TF- α_1 -AT + SAA		
	weight	r	p value	weight	r	p value
Haemoglobin (Hb, g/dL)	0.00	-0.06	0.83	0.00	0.58	0.02*
Leukocyte count ($\times 10^9/L$)	0.40	-0.53	0.04*	0.00	0.35	0.19
Neutrophil count ($\times 10^9/L$)	0.40	-0.54	0.03*	0.00	0.29	0.27
Lymphocyte count ($\times 10^9/L$)	1.0	0.52	0.04*	0.70	0.56	0.02*
Monocyte count ($\times 10^9/L$)	1.00	-0.37	0.17	0.00	0.66	0.00*
Platelet count ($\times 10^9/L$)	0.80	-0.10	0.72	0.00	0.11	0.70
CRP	0.80	0.16	0.58	0.10	0.55	0.04*
ALT (U/L)	0.70	-0.14	0.62	0.00	0.57	0.02*
Creatinine ($\mu M/L$)	0.40	0.20	0.47	0.00	0.21	0.45
CPK	0.90	0.95	0.00*	0.90	0.96	0.00*
Na	0.40	-0.53	0.03*	1.00	-0.15	0.59
K	1.00	-0.40	0.13	1.00	-0.45	0.17
Cl	0.70	-0.47	0.06	1.00	-0.41	0.12

* Significant correlations, see Section 3.4

of TF- α_1 -AT but not with total serum α_1 -AT concentration in the sera from SARS patients, suggesting that TF- α_1 -AT may serve as a valuable biomarker to predict the effectiveness of or the responsiveness to the treatment.

The complement system is involved in many diseases. C4, C3a and C5a are classified as anaphylatoxins because they induce the release of mediators from mast cells which cause the rapid increases in vascular permeability characteristic of anaphylaxis. Our data showed there is an increased level of C4 in SARS patients. Therefore, C4 may be activated or increased in response to SCV. SAA proteins are elevated as part of the response to various injuries, including trauma, infection, inflammation and neoplasia [18]. An increased level of plasma SAA has been linked to the pathophysiology of many chronic inflammatory diseases, but the mechanisms of such induction remain elusive. We showed that there was a higher level of SAA in SARS patients, which was further elevated after treatment. Moreover, our data showed that one of the isoforms for SAA appeared to be missing in sera from healthy controls and patients with nonSARS pneumonia, suggesting it may be associated solely with the SARS patients.

5 Concluding remarks

In conclusion, we have shown that the proteomic approach can generate novel diagnostic and therapeutic molecular markers. It should be noted that measuring serum protein levels is technically much easier than other approaches so far developed, and offers an economical and effective initial detection tool in a clinical setting. It is our hope that validation of these new serological profiles in SARS patients will enable the disease to be detected earlier and provide information on disease-associated targets and the process of pathogenesis. Ultimately, it

also offers hope for the development of molecularly-targeted therapeutics against SARS.

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