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Proteomic profiling in SARS: diagnostic and prognostic applications

Key Messages

1. Disease-specific proteomic fingerprints were found in SARS patients.
2. The two proteomic features yielding the largest receiver operating characteristic curve area (diagnostic accuracy of >95%) were an N-terminal fragment of complement C3c α-chain (m/z 28119) and an internal fragment of fibrinogen alpha-E chain (m/z 5908).
3. In contrast to previous proteomic studies, we found that serum amyloid A was not useful in the diagnosis of SARS.
4. The potential prognostic features of m/z 7768 and m/z 8865 were found to be platelet factor 4 and beta-thromboglobulin, respectively.

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Introduction

Advances in proteomics have provided new strategies to identify biomarkers and therapeutic targets, and to study the pathology of diseases. Surface-enhanced laser desorption/ionisation (SELDI) ProteinChip technology is a proteomic tool that has been applied to the discovery of diagnostic proteomic fingerprints for various diseases, including cancer and infectious diseases.[1-3] This technology has been used to identify potential biomarkers for early diagnosis of SARS.[1,4-6] In these studies, the controls were either healthy subjects or persons with non-SARS viral infection. Regrettably, the similarity of the symptoms between SARS and control patients, and the time point of blood collection were not considered. From the perspective of infectious disease diagnosis, one should identify the disease causing the symptoms in patients presenting with similar symptoms, not differentiate healthy subjects from infected patients.[7]

We compared the serum proteomes between SARS and non-SARS patients, and identified the potential protein marker for diagnosis and prognosis of SARS. The non-SARS patients were those who had similar symptoms to SARS patients. They were admitted to the same hospital and were later shown to be negative for SARS-CoV infection. For both SARS and non-SARS patients, sera were collected within 1 week of the fever onset.

Aims and objectives

1. To characterise the proteomic fingerprints of SARS or specific proteomic features in serum of SARS patients;
2. To investigate if the serum proteomic profiles are useful in early diagnosis of SARS;
3. To investigate if the variations of the serum proteomic profile correlate with clinical events;
4. To investigate if the serum proteomic profiles are of prognostic significance in SARS patients; and
5. To uncover the protein identity of serum proteomic features with potential diagnostic and prognostic value.

Methods

Patients

The SARS group included 13 males and 26 females; the mean age was 42 (range, 21-88) years. The non-SARS group included 18 males and 21 females; the mean age was 44 (range, 20-88) years. The pre-treatment serum samples from both groups represented the first time point after hospitalisation (3-7 days from onset of fever). All the SARS cases were positive for anti-SARS-CoV IgG antibody. The non-SARS patients were controls who had similar symptoms as the SARS patients and were admitted to the same hospital and later shown to be serologically negative for anti-SARS-CoV antibody even 6 weeks after the onset of symptoms.

Serum proteomic profiling

For all the SELDI ProteinChip analyses, the serum samples from the diseased and control groups were randomised and the investigator was blinded. The SELDI ProteinChip analysis was performed as previously described,[2,4,7] using CM10

ProteinChip arrays (Ciphergen Biosystems). Two binding conditions were performed: at pH 4.0 and pH 9.0.

Bioinformatic analysis

The significance analysis of microarray (SAM) algorithm (Stanford University, CA, US) was used to identify proteomic features with levels significantly different between the SARS and non-SARS patients.[2,4,7] Correlations between the differential proteomic features and various clinical and biochemical features were examined by the Spearman rank-order correlation test. Significantly differential proteomic features correlated with various clinical/biochemical correlations were then subjected to two-way hierarchical clustering analysis, as previously described.[2]

Protein purification

For protein identification, proteins corresponding to the SEDLI peaks were purified by cation exchange chromatography with the use of CM10 ceramic beads (BioSepra) under the binding conditions similar to those for CM10 ProteinChip arrays. The purified proteins were resolved by two-dimensional gel electrophoresis. Protein spot with mass matched with the differential proteomic feature was excised and subjected to mass spectrometry (MS) analysis.

Protein identification

Protein spots of interests were removed from the gel and subjected to trypsin digestion as previously described.[8] The trypsin digests were then extracted and subjected to tandem MS analysis using the ABI 4700 system (Applied Biosystems). 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 determine the protein identities.

Results

Identification of differential serum proteomic features

The serum proteomic profiles of 39 SARS and control patients were obtained, and 820 common proteomic features were found. At a median false discovery rate of zero (SAM analysis), levels of 107 serum proteomic features were significantly different between the SARS and control patients. In SARS patients, 52 and 55 proteomic features were present at higher and lower levels, respectively. Among these 107 differential proteomic features, 20 yielded significant correlations with two or more clinical/biochemical parameters. As a result, there were 20 potential biomarkers for the detection of SARS; in SARS patients 15 and 5 yielded positive and negative correlations, respectively. Hierarchical clustering analysis showed that these 20 biomarkers contained information to identify SARS patients at high accuracy (sensitivity=95%, specificity=100%), SARS patients with a poor prognosis (ie requiring care in the intensive care unit or supplementary oxygen).

Correlation with clinical/biochemical parameters

The biomarker of m/z 24504 correlated positively with SARS coronavirus load, whereas that of m/z 4680 correlated negatively with viral load. Ten biomarkers correlated positively with C-reactive protein, suggesting their levels were affected by the acute phase reaction response. Whereas 12 others correlated positively with lactate dehydrogenase levels, which suggested they were associated with the lung damage. Two biomarkers correlated positively with serum albumin and/or total protein levels, indicating an association with the liver function. Whereas 13 others correlated negatively with albumin and/or total protein (but not alanine transaminase), reflecting the effect of decrease in liver function, but their presence may not have been due to liver damage. Three biomarkers correlated positively with age. Ten biomarkers correlated positively (one negatively) with neutrophil counts.

Diagnostic values of the proteomic biomarkers

Receiver operating characteristic (ROC) curve analyses showed that all the differential proteomic features were potential biomarkers for identifying SARS patients. The ROC curve areas of all the 20 biomarkers were in the range of 0.733 to 0.955. For example, the ROC curve for the peak intensity of biomarker m/z 28120 was 0.987 (95% confidence interval [CI], 0.966-1.007). At a specificity of 97%, its sensitivity was 97%. The ROC curve for 1/peak intensity of biomarker m/z 5908 was 0.995 (95% CI, 0.985-1.004). At a specificity of 95%, its sensitivity was 100%.

Analysis of the diagnostic value of serum amyloid A

This SELDI proteomic feature corresponding to serum amyloid A was not identified to be a potential diagnostic marker. This finding was confirmed by immunoassay.

Prognostic values of the proteomic biomarkers

By multivariate logistic regression, we analysed the prognostic values of the 20 SARS-associated proteomic features and 10 serological variables (alanine transaminase, lactate dehydrogenase, bilirubin, total protein, albumin, globulin, C-reactive peptide, total white blood cell count, lymphocyte count, and neutrophil count) in pretreatment samples from 38 SARS patients. Serum proteomic features of m/z 6634 (P=0.010), m/z 7768 (P=0.017) and m/z 8865 (P=0.045) were significantly associated with supplemental oxygen usage by the patients, whereas a proteomic feature of m/z 8635 (P=0.016) was associated with admission to intensive care units.

Purification and identification of the proteomic biomarkers with diagnostic/prognostic values

The proteins corresponding to the differential proteomic features were purified and separated by chromatographic and gel electrophoresis techniques. The purified proteins were subjected to mass spectrometric analysis to identify the proteins. Protein identities of six diagnostic and prognostic proteomic features were obtained (Table).

Table. Protein identities of six diagnostic and prognostic proteomic features using surface-enhanced laser desorption/ionisation (SELDI) ProteinChip technology

SELDI peak (m/z)	Protein identity	SwissProt entry no.	Higher/lower levels in SARS than control patients
5908	Internal fragment of fibrinogen alpha-E chain	P02671	Lower
7768	Platelet factor 4	P02776	Lower
8865	Beta-thromboglobin	P02775	Higher
24500	Ig Kappa light chain	223335 (NCBI)	Higher
28120	N-terminal fragment of complement C3c	P01024	Higher
88650	Immunoglobin heavy constant gamma 1	P01857	Higher

Discussion

Two studies reported potential biomarkers in the sera of adult SARS patients using the SELDI ProteinChip technology.[1,5] In the present study, the intensity of the proteomic feature of m/z 7769 was significantly lower in SARS patients (Mann Whitney test, $P<0.001$), as noted in another study (Mann Whitney test, $P=4.9 \times 10^{-8}$).[1] Other SARS-associated proteomic features differed, probably due to different selection criteria for the control subjects. In previous studies, the controls were either healthy subjects or patients from other clinics with viral infections. The degree of similarity of the symptoms between SARS and control groups, and the time point of blood collection were not considered. In the present study, the controls were suspected SARS patients admitted to the same hospital as SARS patients, but later shown to be negative for SARS-CoV infection. The symptoms and the time points for blood sampling were similar in SARS and control patients. Thus, the biomarkers identified in the present study may be more advantageous in actual diagnostic settings than those identified in previous studies.

The different findings reported in various studies could also be due to the use of different profiling methodologies. In a previous study, a comprehensive profiling approach was used.[1] After denaturing with urea and detergent, the serum proteins were first fractionated with anion exchange beads to give six fractions, which were later analysed with arrays involving copper ProteinChips and weak cation exchange CM10 ProteinChips. The comprehensive profiling approach increases the chance of identifying more potential protein markers.[2] In the present study, we analysed the serum proteins directly, using only the CM10 ProteinChip arrays at two different binding conditions (pH 4 and pH 9). We chose the CM10 ProteinChip arrays (previously called WCX2) because its chip type was shown to give the best profiling when analysing serum samples from the SARS patients.[5] Although the direct binding approach might lead to the discovery of fewer biomarkers, such assays have a higher potential for modification for a clinical assay even without knowing the protein identities of the disease-specific SELDI peaks.

In previous studies, platelet factor 4 (PF4) and beta-thromboglobin (beta-TG) were found to be chemokines involved in the pathogenesis of acute respiratory distress syndrome (ARDS) in a negative and positive manner, respectively. The computed tomographic features of ARDS caused by SARS are similar to those ARDS caused by other causes. In SARS patients, low serum levels of PF4 and high serum levels of beta-TG were associated with a poor prognosis. PF4 and beta-TG may be important chemokines involving the development of ARDS in SARS patients.

Conclusions

Specific proteomic fingerprints were present in the sera of adult SARS patients. They could be used to identify SARS cases during early onset of the disease with high specificity and sensitivity, and could also be used for prognosis. The proteins with potential diagnostic and prognostic values were successfully identified. The SELDI ProteinChip assay could be used for first-line detection of SARS, followed by a quantitative viral RNA assay for confirmation. Once confirmed, the treatment strategy could be adjusted according to the anticipated prognosis, based on the SELDI ProteinChip profiling and the viral RNA level. As the protein identities of the proteomic features with diagnostic and prognostic values have been identified, in the future specific immunoassays may be developed for the diagnosis of SARS and to offer a prognosis.

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