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Detection of potential protein biomarkers with Surface Enhanced Laser Desorption Ionization-Time of Flight-Mass Spectrometry useful in the diagnosis and follow-up of sarcoidosis

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Introduction

Surface Enhanced Laser Desorption Ionization-Time of Flight-Mass Spectrometry (SELDI-TOF-MS) is a promising new technology first introduced by Hutchens and Yip (1). The ProteinChip system manufactured by Ciphergen Biosystem Inc. (Fremont, CA) indicated as SELDI-TOF-MS (PBSII) has the potential to discover useful biomarkers faster than any existing technology. The true scientific goal of serum proteomic pattern analysis was in fact biomarker discovery. However, since the study of Petricoin et al. (2) on proteomic patterns to detect ovarian cancer, the use of SELDI protein profiling as a diagnostic tool, has become an important subject of investigation. Until now this approach has been suggested for different diseases, for example ovarian (2-4) and prostate (5-8) cancer research, but also inflammatory diseases (9).

The focus of the presented study was the detection of potential protein biomarkers in diagnosis of sarcoidosis. In young adults, pulmonary sarcoidosis is the second most common respiratory disease after asthma. Sarcoidosis is a systemic granulomatous disease that primarily affects the lungs and the lymphatic system. The cause of the disorder is still unknown (10). Especially adults under the age of 40 and certain ethnic and racial groups are affected with this disease. Till now there is no good marker for both diagnosis and prognosis of sarcoidosis.

The aim of the present study is to detect potential biomarkers for the diagnosis of sarcoidosis using serum protein profiling by SELDI-TOF-MS and to find out if these specific protein profiles can differentiate sarcoidosis appropriately from diseases with a similar clinical presentation.

Methods

Serum of sarcoidosis and healthy control samples were collected and analyzed on weak cation exchange (CM10) and on normal phase (NP20) ProteinChip arrays (Ciphergen Biosystems, Inc., Fremont, CA). For the CM10 experiment, protein profiles were obtained from serum samples of 40 sarcoidosis patients and 46 healthy persons. Serum samples were denaturated with 9M urea/2% Chaps. For the NP20 experiment, serum samples were fractionated by centrifugation of the serum samples on a 30 kDa cut-off filter. This modified method was used, because highly expressed proteins such as human serum albumin (HSA) and immunoglobin (Ig) interferes with the detection and identification of less abundant proteins, which could be potentially relevant for diagnosis. Therefore it is important to remove major proteins such as HAS and Ig from the serum prior to SELDI-TOF analysis. For this purpose, crude serum of 16 sarcoidosis patients and 16 healthy persons was fractionated by centrifugation of the serum samples on a 30 kDa cut-off filter. Hereafter, the fraction which passed through the filter (filtrate) was applied on NP20 array. The same experiment was repeated using denaturing (9M urea/2% Chaps) conditions where after dialysis was required to remove the added urea before filtration.

Before SELDI-TOF-MS analysis, sinapinic acid matrix was added on the spots of the CM10 and NP20 arrays for the ionization process. All of the ProteinChip arrays were read on a Protein Biological System II ProteinChip reader (Ciphergen Biosystems, Inc., Fremont, CA). External calibration of the instrument was performed using the All-in-1 peptide molecular mass standard (Ciphergen Biosystems, Inc., Fremont, CA).

Data analysis

For the detection of sarcoidosis biomarkers, we compared sarcoidosis samples versus healthy control samples. Peaks were identified after mass calibration, background subtraction, and normalization using the clustering and alignment function of Ciphergen Express Data manager 2.1 (Ciphergen Biosystems, Inc., Fremont, CA). The settings were used as follows: signal/noise ratio in the first pass: 5.0 and signal to noise ratio in the second pass: 3.0, minimum peak threshold 50%, cluster mass window: 0.3%. The part of the spectrum with *m/z* values <1000 Da was not used for analysis, as the energy absorbing matrix signal generally interfered with peak detection in this area. The peak intensities are then transferred to Biomarker Pattern's software (Ciphergen Biomarker Patterns 5.0, Ciphergen Biosystems Inc, Fremont, CA, USA). Peak recognition and sample classification were performed with the Patterns Software. Multiple trees were initially generated with use of the peaks as

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variables. Proteins which showed highly significant differences in peak intensity (Mann-Whitney Wilcoxon test (U-test)) were used for tree-building algorithms to obtain the best classification models. The peaks that formed the main splitters of the tree(s) with the highest prediction rates in the cross-validation analysis were then selected and used to make a final decision tree with the greatest possible predictive power. The correct classified percentages for both groups were calculated after the cross validation.

Table 1. Results of tree-building algorithms with Biomarker Patterns Software. The discriminating peaks (*m/z* values) including mean intensity (Int) and the number (N) and percentages (%) correct classified of the sarcoidosis and healthy control samples on CM10 and NP20 arrays are illustrated in this table.

	m/z	Int	*m/z*	Int	*m/z*	Int	N correct	% correct
Denatured serum (CM10)								
Sarcoidosis	3808	12.5	4277	10	8932	11	31/34	92
Healthy control	3808	5	4277	25	8932	6	35/39	90
Filtrated crude serum (NP20)								
Sarcoidosis	2454	1.0					16/16	100
Healthy control	2454	32.0					14/16	88
Filtrated dialyzed serum (NP20)								
Sarcoidosis	4287	2.5					15/16	94
Healthy control	4287	25.0					15/16	94

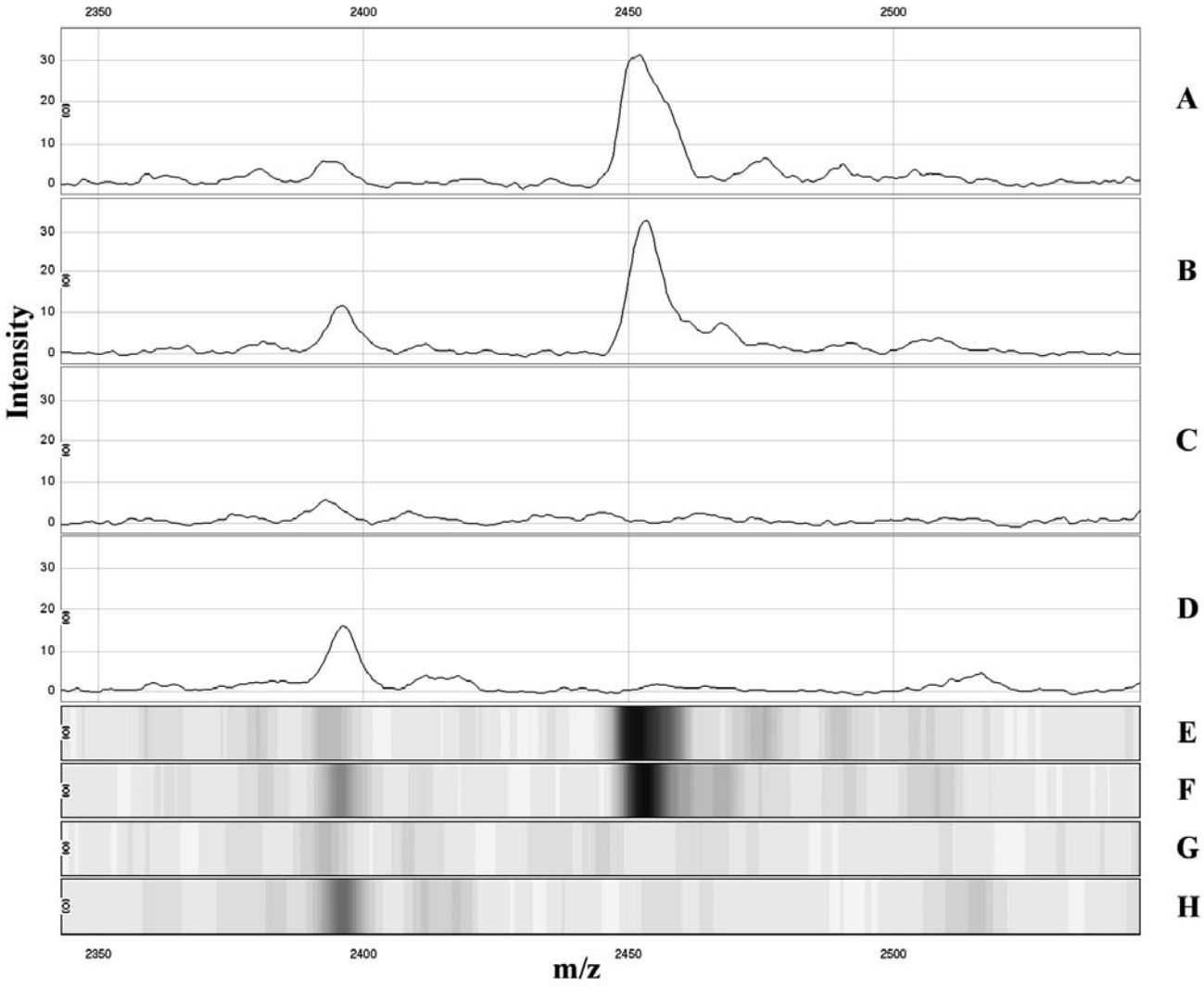

Figure 1. SELDI mass spectra and gel views of fractionated serum samples by centrifugation on a 30 kDa cut-off filter on a NP20 array. Panel A and B show spectra of two healthy control samples. Panel C and D show spectra of two sarcoidosis patient samples. Panel E and F show healthy control spectra in gel view and panel G and H show sarcoidosis spectra in gel view. The intensity is displayed along the y-axis and the mass is given as mass/charge ratio (m/z) ratio on the x axis.

Results

Three peaks were found on the CM10 arrays to discriminate sarcoidosis from healthy control samples (m/z values: 3808 Da (up-regulation in sarcoidosis), 4277 Da (down-regulation in sarcoidosis), 8932 Da (up-regulation in sarcoidosis). A 10-fold cross validation was performed to assess the algorithm's ability to predict between the groups with resulting correct classified percentages of 91% and 90% for sarcoidosis and healthy controls, respectively. A single peak was found on the NP20 ProteinChip array for both filtrated crude and dialyzed serum samples to discriminate sarcoidosis from control samples, namely *m/z* 2454 Da and m/z 4287 Da for crude and dialyzed serum respectively. A 10-fold cross validation was performed with resulting correct classified percentages of 100% and 88% for sarcoidosis and healthy controls respectively in filtrated crude serum. Filtrated crude serum samples were down-regulated in the sarcoidosis compared to the healthy control group with mean intensities of 1.0 and 32.0 respectively. In filtrated dialyzed serum the correct classified percentages were 94% for both groups. Filtrated dialyzed serum samples were also down regulated in the sarcoidosis samples compared to healthy control samples with mean intensities of 2.5 and 25.0 respectively (table 1).

Figure 1 illustrates SELDI mass spectra and gel views of the single peak found on the NP20 arrays with filtrated crude serum samples of 2 sarcoidosis patients and 2 healthy controls. The peak at m/z 2454 Da is clearly down-regulated in the sarcoidosis compared to the healthy control samples.

Conclusion

This study acts as a proof-of-principle for the use of SELDI-TOF in the investigation of sarcoidosis. Further research is needed to detect specific sarcoidosis biomarkers, eventually followed by implementation of a quantitative assay, to discriminate sarcoidosis from diseases with a similar clinical presentation.

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