Chapter 6

Potential biomarkers for diagnosis of ankylosing spondylitis using SELDI-TOF-MS

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Abstract

Background
Ankylosing spondylitis (AS) is a chronic systemic inflammatory rheumatic disorder that primarily affects the axial skeleton, with sarcoiliitis as its hallmark. It has been estimated that at least 30% of patients do develop severe spinal restriction during the natural course of the disease. Until now there is no satisfying biomarker for diagnosis or prognosis of AS. This study is focused on the detection of potential biomarkers for the diagnosis of AS using Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS).

Methods
Sera of 38 AS patients and 38 healthy controls were used to detect potential biomarkers. Serum was separated using an anion exchange fractionation procedure. In the screening experiments, three ProteinChip array surfaces; cation exchange (CM10), metal affinity binding array coupled with copper (IMAC-Cu^{2+}), and hydrophobic, were compared to find out which condition resulted in the optimal discrimination of both groups.

Results
The optimal discrimination was reached with the following conditions; CM10 array with the organic serum fraction and IMAC-Cu^{2+} array with denatured serum. Analyses of all AS and healthy control samples on CM10 arrays resulted in a sensitivity of 66% and a specificity of 74% using a multimarker approach of two peaks. The peak at *m/z* 4172 was used as first splitter in the decision tree and was up-regulated in the AS group and the peak at *m/z* 28,144 was used as second splitter. Analyses of all AS and healthy control samples on IMAC-Cu^{2+} arrays resulted in a sensitivity and specificity of both 70% using a multimarker approach of two peaks. The peak at *m/z* 6644 was used as first splitter and was down-regulated in the AS group and the peak at *m/z* 13,875 was used as second splitter. The peaks at *m/z* 28,144 and 13,875 were both successfully identified as apolipoprotein A-I.

Conclusions
This is the first study that shows that protein profiling in serum using SELDI-TOF-MS can be used as a diagnostic tool for AS. In future studies, the sample group will be enlarged and we will also validate our markers with a blind sample set.

Introduction

Ankylosing spondylitis (AS), a frequent, chronic, inflammatory rheumatic disease, is the prototype and the most severe form of the spondylarthritides. AS affects young patients, predominantly males, most frequently starting in the third decade of life, with a prevalence of 0.1-1.1%[1]. AS is characterized by spinal inflammation with sacroiliitis, spondylitis, spondylodiscitis, and spondylarthritis, but also by new bone formation with syndesmophytes and ankylosis[2]. It has been estimated that at least 30% of patients do develop severe spinal restriction during the natural course of the disease[3]. As in most rheumatic diseases, the severity of symptoms varies greatly among patients and varies over time in individual patients[4]. It may lead to major functional limitations not only because of spinal disease but also because of extraspinal disease manifestations such as peripheral arthritis, inflammatory bowel disease and uveitis[5].

Spinal structural damage can be assessed on conventional radiographs as destructive and proliferative lesions ultimately leading to syndesmophyte formation. There is a wide variation in the amount of damage among patients ranging from no spinal damage to complete bamboo spine. However, the underlying pathogenetic mechanisms remain largely unknown. With proteomics analysis, potential biomarkers can be detected which can possibly improve the understanding of the underlying pathogenetic mechanisms of AS. Proteomics analysis using Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS) has been successfully employed in the discovery of new biomarkers in different human diseases[6]. SELDI-TOF-MS is a technology first introduced by Hutchens and Yip[7]. One of the key features of SELDI-TOF-MS is its ability to provide a rapid protein expression profile from a variety of biological and clinical samples[8]. It consists of selective protein extraction and retention on chromatographic chip surfaces and their subsequent analysis by a simple laser desorption ionization mass spectrometer[9]. For the detection of potential biomarkers, protein profiles of different groups are compared to find distinctive patterns which can clearly discriminate the different groups. Till now this was mainly applied in cancer[10-13] and chronic inflammation[14-16] research to find biomarkers.

To our knowledge, this proteomics approach using SELDI-TOF-MS has never been applied in AS. The present study is focusing on the detection of potential biomarkers for the diagnosis of AS using SELDI-TOF-MS. It might also provide new insight in the underlying pathological mechanism. In future studies, we will enlarge our sample set and we will also search for biomarkers which indicate the severity of the disease.

Materials and methods

Blood samples of patients with AS (n=38) and healthy controls (n=38) were analyzed. The study was performed in accordance to the Declaration of Helsinki and its amendments and the Medical Ethical Committee of the Hospital approved the procedure followed. Informed consent was obtained from all participants. Characteristics of the studied population are illustrated in Table 6.1. Collection of serum samples was standardized according to World Healthy Organization (WHO) recommendations on anticoagulants in diagnostic laboratory investigations (2002) by using a clotting time of 30 min at room temperature, spinning for 15 min (3000 g) and storage of the samples in aliquots within one hour at –80°C after blood collection. All fractionated serum samples were thawed only twice from collection to analyzing.

During screening experiments, sera of eight unrelated human leucocyte antigen B-27 (HLA-B27) positive AS patients and sera of eight healthy controls were used to determine the optimal conditions. The HLA-B27 positive AS patients had significant spinal damage and radiological progression over the past two years determined with the modified Stoke Ankylosing Spondylitis Spine Score (mSASSS) method[17].

Table 6.1 Patient characteristics.

	Ankylosing spondylitis patients	Healthy controls
n	38	38
Gender, M/F	29 / 9	18 / 20
Age, years	47.5 ± 10.5	46.4 ± 10.9
Disease duration since diagnosis, years	10.5 ± 8.4	
mSASSS baseline	18.5 ± 17.7	
HLA B27-haplotype, M/F	29 / 7	
CRP (mg/dl)	13.2 ± 12.2	
ESR (mm/first hour)	11.5 ± 6.7	
BASMI (10-point scale)	4.2 ± 1.5	
BASDAI	3.6 ± 2.0	
BASFI	4.2 ± 2.2	
NSAID use, M/F	29 / 9	

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, BASFI: Bath Ankylosing Spondylitis Functional Index, BASMI: Bath Ankylosing Spondylitis Metrology Index (BASMI), CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, HLA-B27: human leucocyte antigen B-27, mSASSS: modified Stoke Ankylosing Spondylitis Spine Score, NSAID: Non-Steroidal Anti-Inflammatory Drugs.
Variables presented as mean ± SD, as they follow a Gaussian distribution.

SELDI-TOF-MS analysis

An anion exchange procedure was used, which allowed high-throughput fractionation of serum as described by Solassol et al.[18]. Serum was separated into six different fractions (pH9, pH7, pH5, pH4, pH3, and organic wash). The anion exchange fractionation was standardized according to the Bio-Rad (Bio-Rad Laboratories, Inc., Herculus, CA, USA) protocols. For the anion exchange

fractionation, we used 20 µl serum and 30 µl (9M urea, 2% Chaps, 50 mM Tris-HCL pH9) as starting material. During the screening experiment the denatured serum samples and the six fractions of the eight unrelated HLA-B27 positive AS patients and of the eight healthy unrelated controls were applied to three different ProteinChip array surfaces (Bio-Rad); cation exchange (CM10), metal affinity binding coupled with copper (IMAC- Cu^{2+}), and hydrophobic (H50) to find out which condition resulted in the best discrimination of both groups. The CM10, IMAC-Cu^{2+} and H50 experiments were performed according to the Bio-Rad protocols. The ProteinChip arrays were read on a Protein Biosystem IIc (Bio-Rad) instrument. A well defined protocol for calibration of the PBS IIc, implementation of quality control (QC) samples and acceptance criteria for QC were used in this study[19].

Data analysis

For the detection of AS biomarkers, serum protein profiles of AS patients and healthy controls were compared. Peaks were auto detected using Ciphergen Express Data manager 3.0.6 (Bio-Rad). The biomarker Wizard parameters were as followed; signal-to-noise ratio (S/N) for the first pass: 5.0 (peak height) and 3.0 (valley depth) and for the second pass: 3.0 (peak height) and 1.0 (valley depth). The minimal peak threshold was 50% of all spectra. The cluster mass window was set at 1.0 peak width and the specified *m/z* range was 2000 to 30,000 for the low range, 5000 to 100,000 for the mid range, and 10,000 to 200,000 for the high range. The Mann-Whitney Wilcoxon test (U-test)) was used to detect significant differences in the intensities of the *m/z* values between both groups. The biomarker wizard clusters were exported to Biomarker Patterns (BPS) 5.0.2 (Bio-Rad).

BPS uses the peak information generated by the training set of known samples to build a binary decision tree algorithm. The algorithm functions by assigning each sample in the data set into one of the two groups or nodes with a rule based on the intensity of a particular peak or splitter. Each sub node has a different rule that further divides the data set and this process continues until all cases are assigned into terminal nodes. This results in correct classification percentages of the so called “learn set”. The software generates and tests the models, using a process of cross-validation by randomly picking 10% of the samples. The peaks that formed the main splitters of the tree with the highest prediction rates in the cross-validation analysis were then selected to make a final decision tree with the greatest possible predictive power and this results in correct classification percentages of the so called “test set”. So the classification ability of the decision tree algorithms in the learning set to distinguish between AS and controls was validated after cross-validation, which results in a test set.

A Bayesian approach was used to calculate the expected probabilities of each class in each terminal node. Sensitivity was calculated as the ratio of the number of correctly classified disease samples to the total number of disease samples.

Specificity was calculated as the ratio of the number of control samples correctly classified to the total number of control samples[12].

Identification

Protein identification was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% sodium dodecyl sulphate polyacrylamide (Bio-Rad) and 20-25% gradient sodium dodecyl sulphate polyacrylamide gels followed by MALDI-TOF-MS. To identify a biomarker, samples were chosen which presented a clear up- or down-regulation in the protein spectra. Ten µl of denatured serum or fraction were diluted in 90 µl Mi-Q. Ten µl of these diluted serum samples were subsequently diluted with 2 µl 10% SDS sample buffer (312.5 mmol/l TRIS-HCl, 10% SDS, 33% glycerol, 0.01% bromophenol blue) and loaded on 12.5% sodium dodecyl sulphate-polyacrylamide gels. For the 20-25% gradient sodium dodecyl sulphate polyacrylamide gels, 10 µl of denatured serum or fraction were diluted in 15 µl Mi-Q plus 15 µl 4% SDS sample buffer (62.5 mmol/l TRIS-HCl, 4% SDS, 22% glycerol, 0.01% bromophenol blue). The digestion procedure was performed as described in the studies of Bouwman et al.[20,21] with minor modifications.

MALDI-TOF-MS

The MALDI-TOF-MS analysis was performed as described in the studies of Bouwman et al.[20,21] with minor modifications. For Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (MALDI-TOF-MS), 1.5 µl of each peptide mixture and 0.5 µl matrix solution (2.5 mg/ml CHCA in 50% acetonitrile/0.1% trifluoroacetic acid (TFA)) were spotted automatically onto a 96-well-format target plate. The spots were allowed to air dry for homogeneous crystallization. Spectra were obtained using a M@LDI-LR mass spectrometer (Waters, Milford, MA, USA). The instrument was operated in positive reflector mode. The peptide mass list was searched with ProteinLynx Global Server v2.0 (Waters) or Mascot search engine against the Swiss-Prot database. One miss-cleavage was tolerated, carbamidomethylation was set as a fixed modification and oxidation of methionine as an optional modification. The peptide mass tolerance was set to 100 ppm. No restrictions were made on the protein M_r and the pI. A protein was regarded as identified when it had a significant ProteinLynx or Mascot probability score ($p<0.05$), a minimum of four peptides were required to match.

Results

Screening experiment

For choosing the optimal fraction and array type, the number of significant mass-to-charge (*m/z*) values and the cluster plots were evaluated for each condition with the Ciphergen Express software. For single charged molecules, the *m/z* value is equal to the molecular weight (MW) in dalton (Da). The cluster plots illustrate the distribution of the intensities for each *m/z* value. With an increased number of significant *m/z* values ($p<0.05$) and superior cluster plots, the chance to detect a potential biomarker in a larger sample set is theoretically higher. A cluster plot is superior when there is only minor or no overlap between the data points of the AS patients compared to the control samples, which means that there is a great absolute difference between the mean intensities of both groups, which results in a good discrimination between both groups. By comparing the different chips and fractions in the screening experiments with eight AS patients and eight healthy control samples, we detected the most significant *m/z* values ($p<0.05$) with superior cluster plots in samples of fraction 6 (the organic fraction on the CM10 arrays (n=5) and in the denatured serum samples (n=6) on the IMAC-Cu^{2+} arrays. These two conditions were used for performing the CM10 and IMAC-Cu^{2+} experiments with all AS and all control samples. The numbers of the significant peaks with a superior cluster plot are illustrated in Table 6.2.

Table 6.2 The numbers of significant peaks with a superior cluster per ProteinChip array and fraction.

Fraction	CM10 ProteinChip arrays	IMAC-Cu^{2+} ProteinChip arrays	H50 ProteinChip arrays
Denatured	3	6	-
Fraction 1 (pH 9)	2	1	-
Fraction 2 (pH 7)	-	-	-
Fraction 3 (pH 5)	2	3	-
Fraction 4 (pH 4)	-	1	3
Fraction 5 (pH 3)	1	1	-
Fraction 6 (organic wash)	5	4	1

SELDI-TOF-MS analysis

Analyses of all AS and healthy control samples on CM10 arrays resulted in a sensitivity of 66% and a specificity of 74% using a multimarker approach of two peaks (*m/z* 4172 and 28,114). The peak at *m/z* 4172 was used as first splitter in the decision tree and was up-regulated in the AS group and the peak at *m/z* 28,144 was used as second splitter (Figure 6.1A). The second splitter was used to further classify the samples, but this peak at *m/z* 28,144 only showed a slight, not significant up-regulation in the AS group. Analyses of all AS and healthy control

samples on IMAC-Cu^{2+} arrays resulted in a sensitivity and specificity both of 70% using a multimarker approach of two peaks (*m/z* 6644 and 13,875). The peak at *m/z* 6644 was used as first splitter in the decision tree and was down-regulated in the AS group and the peak at *m/z* 13,875 was used as second splitter (Figure 6.1B). This second splitter was also used to further classify the samples, but this peak at *m/z* 13,875 only showed a slight, not significant down-regulation in the AS group.

The peak splitters with their cut-off values of the peak intensities, and the correct classified percentages of the learn and test set for AS and healthy control samples for the CM10 arrays and the IMAC-Cu^{2+} arrays are illustrated in Table 6.3 and Table 6.4, respectively. The protein spectra of the *m/z* 4172 peak on the CM10 arrays are shown in Figure 6.2 and the protein spectra of the *m/z* 6644 peak on the IMAC-Cu^{2+} arrays are shown in Figure 6.3.

In the present study we also divided our AS group in subgroups to find some markers for disease severity based on spinal structural damage. The following subgroups were made; damage (n=19) vs non-damage (n=19), progression during the last two years (n=17) vs non-progression (n=21), progression and damage (n=12) vs the rest of the AS group (n=26). A few statistically significant peaks were found between the subgroups, but there was a high proportion of incorrect classification of individual patients and controls using decision tree algorithm.

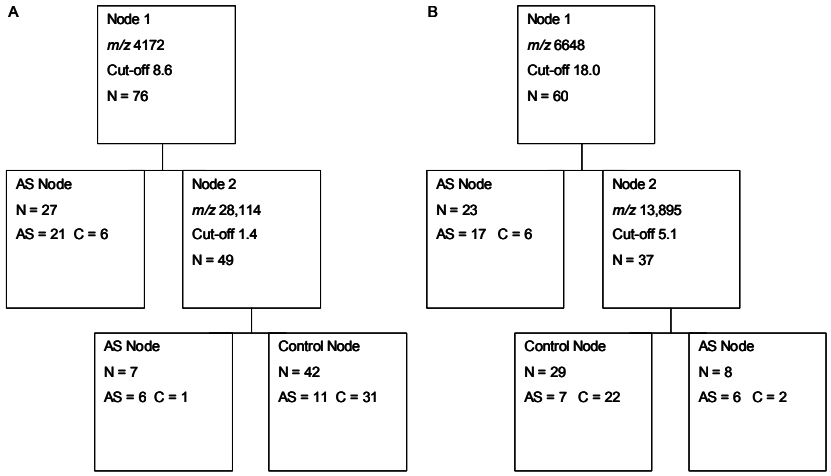

Figure 6.1 Decision trees of the multimarker on the CM10 arrays (A) and on the IMAC-Cu^{2+} arrays (B). The splitter nodes with accessory *m/z* values and cut-off values, end nodes and the number of ankylosing spondylitis (AS) and control (C) per node are illustrated.