Comparative Analysis of Control and Breast Cancer Sera Using a Multidimensional Separation Strategy

Application

Proteomics

Authors

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Abstract

Human serum is frequently used for the discovery of new disease markers, drug targets, or studying protein expression patterns in human biology. However, the presence of high-abundance proteins in serum interferes with the analysis of low-abundance proteins by limiting the dynamic range of mass spectral, electrophoretic, and chromatographic analysis. Reducing sample complexity becomes an important first step in proteomic analysis of complex samples and successful biomarker discovery. We applied a new multidimensional separation technique to reduce sample complexity in the comparative analysis of serum samples from breast cancer patients and healthy control subjects.

Introduction

A high capacity multiple affinity removal column is applied to specifically remove six high-abundant proteins (albumin, IgG, transferrin, haptoglobin, IgA, and anti-trypsin) in human serum. A macroporous reverse-phase C18 (mRP-C18) column was used to fractionate the depleted serum. Under the optimized mRP-HPLC condition, protein recoveries for depleted serum samples were greater than 99%. Reproducibility among patient samples was excellent based on the UV trace overlays. The mRP-C18 fractionated serum samples were further analyzed by 1D-PAGE and followed by in-gel tryptic fragmentation and peptide analysis using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) for selected gel bands. The mRP-C18 fractions were also analyzed by in-solution digestion and two dimensional-liquid chromatography tandem mass spectrometry (2D-LC/MS/MS). The combination of affinity chromatography with high recovery mRP-C18 fractionation and subsequent gel separation reduces sample complexity and greatly increases the number of proteins identified. This multidimensional separation enables the identification of differences in protein expression between healthy and breast cancer serum samples. This new strategy can be adapted to other biological analysis and cancer model studies.

Experimental

Sera

Breast Cancer and Healthy control sera were purchased from Genomics Collaborative (Cambridge, MA).

High Capacity Multiple Affinity Column

An Agilent High Capacity Multiple Affinity Removal Column (Hu6-HC), 4.6-mm id × 50 mm (Agilent part number 5188-5332) was used to

remove the top six proteins from the cancer and control sera. The column was used according to manufacturer's published protocol.

Briefly, serum was diluted 5× with Buffer A, loaded onto the column, and the flow-through collected, pooled, and concentrated. Buffer B was used to elute the bound proteins from the column prior to requilibration in Buffer A.

Protein Fractionation of Flow-through Proteins

The concentrated flow-through fractions from several runs of cancer or control were diluted in 6M acidic urea. The urea denatures the proteins and aids in recovery while the acid prevents carbamylation at the elevated temperatures during separation. An mRP-C18 column, 5 µm, 9.4-mm id × 50 mm (Agilent specially packed and available as custom order, part number 5188-5345) was used to fractionate the proteins. The flow-through proteins were separated under RP conditions on an Agilent 1100 HPLC. Forty-nine fractions were collected under optimized conditions and analyzed by 1D SDS-PAGE, and four fractions from each serum sample were also analyzed by 2D-LC/MS/MS.

SDS-PAGE

Aliquots from the flow-through and bound fractions were run on 4%–20% Tris-Glycine pre-cast gels (Invitrogen). For bound fractions, samples were desalted with 20-mM Tris-HCl. Sample preparation and electrophoresis conditions followed the manufacturer's suggested protocol. Indicated gel spots were cut and processed with Agilent in-gel digestion kit (Agilent part number 5188-2749) and analyzed by MALDI-TOF.

MS Analysis

LC/MS/MS: Protein digests were first fractionated with an SCX column. Each fraction was analyzed with 1D LC/MS/MS using Agilent 300SB-C18, 5 µm, 0.3-mm id × 5 mm as a trap column, and a reverse-phase column SB C18, 3.5 µm, 0.075-mm id × 150 mm, with Agilent 1100 HPLC (300 nm), and MSD Trap XCT. Results were processed by Spectrum Mill software (Agilent Technologies).

Results and Discussion

The ability to fractionate samples at whole protein levels aids in the reduction of sample complexity. The process of RP-separation coupled to UV detection permits, on a broad scale, the ability to compare chromatograms from control samples to those of disease/treated samples for gross differences. This permits a more rapid screening for proteins of interest while all fractions are available for analysis after the initial screen. A comparison of breast cancer serum to control serum is performed here using the Multiple Affinity Removal Column to fractionate high-abundant proteins prior to protein fractionation with the mRP-C18 column.

Figure 1 shows the initial depletion as analyzed by SDS-PAGE gel. No discernable differences can be detected between the control sample and the patient samples from the initial depletion of the six major abundant proteins in serum, including albumin, IgG, IgA, transferrin, anti-trypsin, and haptoglobin.

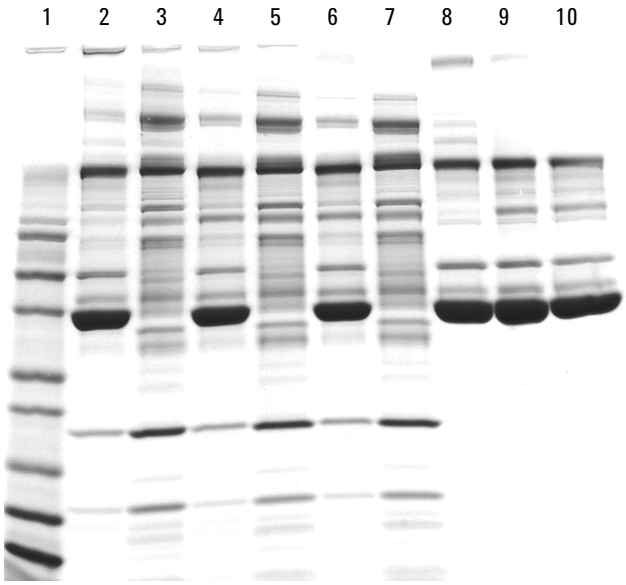

Figure 1. SDS-PAGE analysis of the healthy control and two breast cancer patients' serum fractions before and after immunodepletion. Lane 1, MW markers; Lane 2, control serum, SIW3TSB; Lane 3, control serum flow-through fraction; Lane 4, patient 1, SIDV1SC6; Lane 5, patient 1, SIDV1SC6 flow-through fraction; Lane 6, patient 2, SIDV1S3W; Lane 7, patient 2, SIDV1S3W flow-through fraction; Lane 8, control serum, bound fraction; Lane 9, patient 1 bound fraction; Lane 10, patient 2 bound fraction. A total of 8-µg protein was loaded for each sample.

The flow-through fractions for each sample were prepared according to the methods section prior to injection and fractionation on the mRP-C18 column. The separation was performed at 80 °C using a multistep gradient for optimal recovery and resolution. The chromatograms were staggered and compared for any gross changes. As indicated in Figure 2, there were observable UV differences between the control sample and the patient samples, particularly around fractions 19–28.

Gel-based and in-solution digests were used to analyze each gel band or four mRP-C18 fractions. Fractions from the mRP-C18 column were first analyzed on SDS-PAGE gels for differences in patterns or staining/expression levels (Figure 3). Various protein bands were labeled and excised from the gel, digested and analyzed by LC/MS for identification. In addition, an aliquot from four fractions for control and patient that showed differences was subjected to in-solution tryptic digestion.

Proteins that were identified from in-gel tryptic digests are listed in Table 1. The completeness of removal of haptoglobin was determined to be more than 99% by ELISA for normal control serum.

Haptoglobin is an acute-phase reactant and overexpressed in cancer patients. This is noted by the presence of haptoglobin in the patient samples but not in the control.

Table 1. Proteins Identified from SDS-PAGE Gel Bands by LC/MS

Gel band	Protein(s) identified
1	Histidine-rich glycoprotein
2	Kininogen
3	C4, Synaptonemal complex protein
4	Kininogen, Coagulation factor XIIIB
5	Haptoglobin
6	Histidine-rich glycoprotein
7	Kininogen
8	Hemopexin
9	Serum Amyloid A
10	Complement C8
11	Complement factor B
12	Complement C7
13	Clusterin
14	Complement C4
15	Complement C4, Synaptonemal complex protein

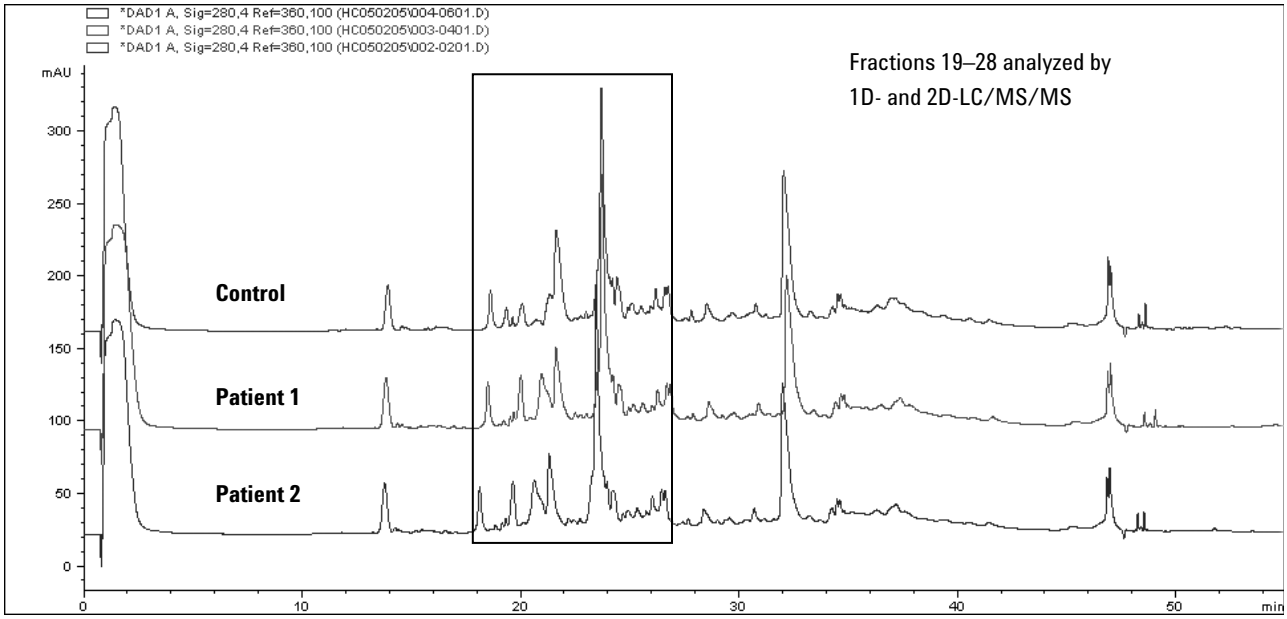

Figure 2. The mRP-C18 chromatograms from control, patient 1, and patient 2 fractionations. The box indicates the fractions (19–28) chosen for initial analysis by LC/MS based on differences within the UV trace.

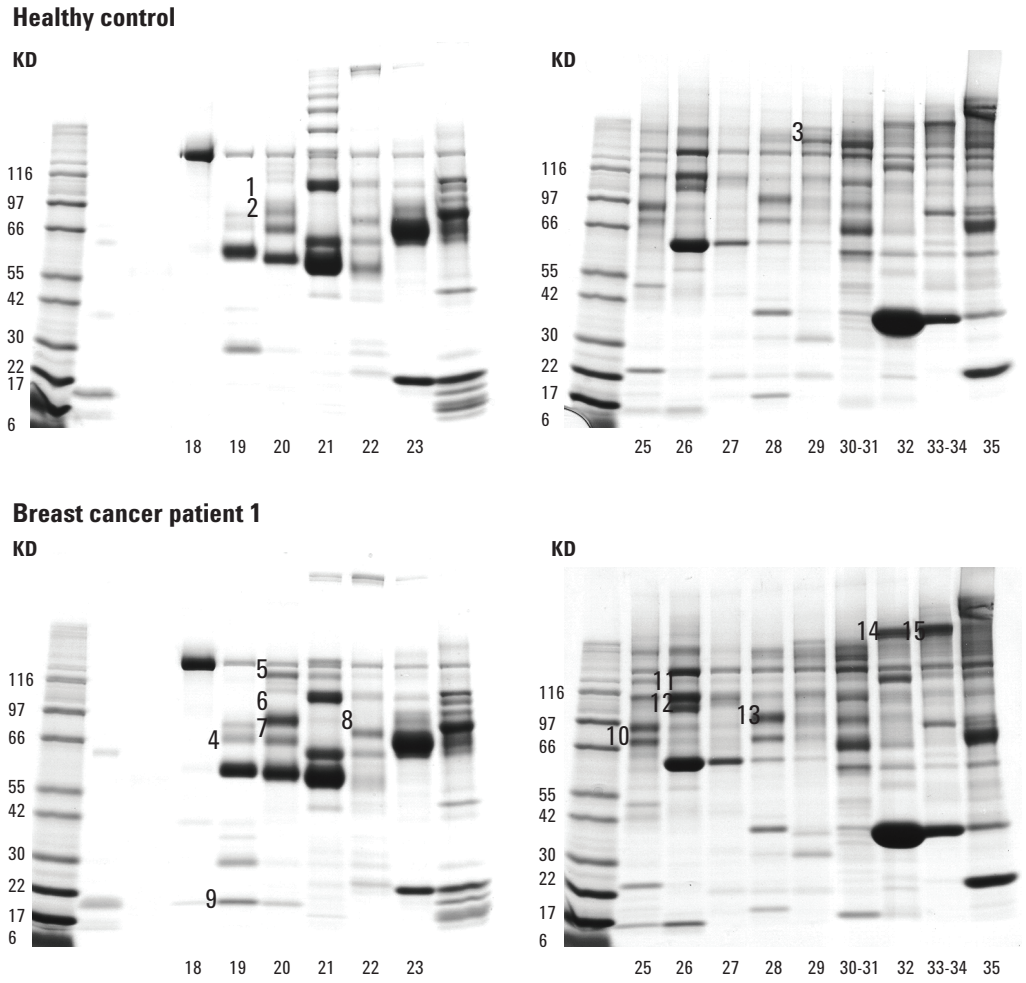

Figure 3. SDS-PAGE analysis of the control and patient mRP-C18 fractions. The numbered gel bands were excised, digested and identified by LC/MS (Table 1).

The analysis of four fractions from the mRP-C18 separation revealed several distinct proteins that only appeared in the control sample (Table 2), only appeared in the patient sample (Table 3), or appeared in both control and patient, though at different levels of expression (Table 4). The results indicate the power of coupling immunodepletion to protein fractionation for the potential identification of biomarkers. The initial prescreen of the UV chromatogram allows one to quickly focus on the gross differences before probing further with the remaining fraction. The ability to recover proteins at >98% when using the mRP-C18 permits the comparison of the immunodepleted serums. Together, the mRP-C18 and multiple affinity columns provide reliable, reproducible and robust performance for biomarker discovery.

Table 2. Proteins Identified in Healthy Control Serum Only

Protein names	NumSpectra*	Numpeps**
Full-length cDNA clone CS0DD006YL02 of neuroblastoma	17	7
Complement C7	5	4
Slice isoform 2 of inter-a-trypsin inhibitor heavy chain H4 precursor	5	3
Tropomyosin 4	3	3
Complement C1r-like proteinase	6	2

*Number of matched MS/MS spectra

**Number of unique peptides

Table 3. Proteins Identified in Cancer Patient 1 Serum Only

Protein names	NumSpectra*	Numpeps**
Complement C4 precursor	16	7
Splice isoform short of complement factor H-related protein 2 precursor	23	4
Complement factor H-related 5	6	5
IGHM protein	8	5
Splice form 2 of attractin precursor	4	4
Clusterin	6	3
Extracellular matrix protein 1 precursor	8	3

*Number of matched MS/MS spectra

**Number of unique peptides

Table 4. Proteins in Both Sera Samples with Different Expression Levels

Protein Names	Healthy control NumSpectra* (Numpep**)	Patient NumSpectra* (Numpep**)	Ratio***
Serum amyloid A protein precursor	8(4)	107(8)	0.08
Cystatin C precursor	1(1)	9(6)	0.2
Lumican precursor	2(1)	8(4)	0.25
Coagulation factor V precursor	2(2)	6(5)	0.33
Selenoprotein P precursor	1(1)	3(2)	0.33
Complement component C8 gamma chain precursor	5(3)	13(4)	0.38
AMBP protein precursor	9(4)	21(6)	0.42
Inter-alpha-trypsin inhibitor heavy chain H2 precursor	8(2)	17(4)	0.47
Hepatocyte growth factor activator precursor	6(2)	12(4)	0.5
Coagulation factor XIIIB chain precursor	7(4)	3(3)	2.3
Splice isoform 1 of complement factor H precursor	260(40)	98(28)	2.65
Plasma retinol-binding protein precursor	80(5)	28(7)	2.85

*Number of matched MS/MS spectra

**Number of unique peptides

***Ratio of control over Patient 1 spectra number

Conclusions

The high-capacity affinity removal column greatly reduces serum complexity by removing the six most-abundant proteins. The ability to fractionate whole protein with the mRP-C18 column allowed for excellent reproducibility and high protein recoveries. The multidimensional separations enable a thorough serum sample comparison from healthy and diseased subjects by 1D SDS-PAGE and 2D-LC/MS/MS. A comparison of proteins identified in the control and patient sera indicates a number of proteins with significant differential expression levels which were identified in these two samples. This multidimensional separation strategy may be used to identify protein biomarkers critical to pathological events and pharmacologic intervention from a variety of sample sources.

For More Information

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