Research Overview

Rapid Protein Display Profiling of Cancer Progression Directly From Human Tissue Using a Protein Biochip

Cloud P. Paweletz,[1,4,10] John W. Gillespie,[2] David K. Ornstein,[3,6] Nicole L. Simone,[4] Monica R. Brown,[5] Kristina A. Cole,[6] Quan-Hong Wang,[7] Jing Huang,[8] Nan Hu,[8] Tai-Tung Yip,[9] William E. Rich,[9] Elise C. Kohn,[5] W. Marston Linehan,[3] Thomas Weber,[11] Phil Taylor,[8] Mike R. Emmert-Buck,[6] Lance A. Liotta,[4] and Emanuel F. Petricoin III[1]*

[1]*Tissue Proteomics Unit, Division of Cytokine Biology, CBER, Food and Drug Administration, Bethesda, Maryland*
[2]*Cancer Genome Anatomy Project, Office of the Director, National Cancer Institute, Bethesda, Maryland*
[3]*Urologic Oncology Branch, National Cancer Institute, Bethesda, Maryland*
[4]*Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland*
[5]*Molecular Signalling Unit, Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland*
[6]*Pathogenetics Unit, Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland*
[7]*Pathology Laboratory, Shanxi Cancer Hospital, Taiyuan, China*
[8]*Cancer Prevention Studies Branch, National Cancer Institute, Bethesda, Maryland*
[9]*Ciphergen Biosystems, Inc., Palo Alto, California*
[10]*Georgetown University, Department of Chemistry, Washington, DC*
[11]*Roswell Park Medical Center, Buffalo, New York*

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ABSTRACT The complicated, changing pattern of protein expression should contain important information about the pathologic process taking place in the cells of actual tissue. Utilization of this information for the selection of druggable targets could be possible if a means existed to rapidly analyze and display changes in protein expression in defined microscopic cellular subpopulations. As a demonstration of feasibility, we show the generation of sensitive, rapid, and reproducible molecular weight protein profiles of patient-matched normal, premalignant, malignant, and metastatic microdissected cell populations from stained human esophageal, prostate, breast, ovary, colon, and hepatic tissue sections through the application of an affinity-based biochip. Reproducible, discriminatory protein biomarker profiles can be obtained from as few as 25 cells in less than 5 min from dissection to the generation of the protein fingerprint. Furthermore, these protein pattern profiles reveal reproducible changes in expression as cells undergo malignant transformation, and are discriminatory for different tumor types. Consistent protein changes were identified in the microdissected cells from patient-matched tumor and normal epithelium from eight out of eight different malignant esophageal tissue sets and three out of three malignant prostate tissue sets. A means to rapidly generate a display of expressed proteins from microscopic cellular populations sampled from tissue could be an important enabling technology for pharmacoproteomics, molecular pathology, drug intervention strategies, therapeutic assessment of drug entities, disease diagnosis, toxicity, and gene therapy monitoring. Drug Dev. Res. 49:34–42, 2000.

Key words: SELDI; proteomics; biomarker; microdissection; laser; pharmacoproteomics; pharmacogenomics; mass spectrometry

Questions concerning the Ciphergen SELDI instrumentation should be directed to Tai-Tung Yip, Ciphergen Biosystems, Inc., Palo Alto, CA.

*Correspondence to: Emanuel F. Petricoin III, Division of Cytokine Biology, CBER, FDA 8800 Rockville Pike, Bldg 29A, Bethesda, MD 20892. E-mail: petricoin@cber.fda.gov

INTRODUCTION

The assignment of biological relevancy to proteins expressed in normal and diseased cells can optimally be achieved if the analysis can be performed in cells from the actual tissue itself. High-throughput intermediate endpoint analysis will be an important adjunct to the assessment of therapeutic intervention and the validation of candidate targets for drug intervention. The application of functional genomics to the study of disease processes directly from human tissue has recently been described [Chuaqui et al., 1997]. In these studies, mRNA levels from many genes were simultaneously measured via high-throughput array analysis. However, a recent study has shown that there is no specific correlation between mRNA abundance and protein expression levels in a cell at a given time [Anderson and Seilhamer, 1997] Alternatively, proteomic biology attempts to explain disease and the effects of therapeutic intervention strategies on the diseased cell populations in terms of the relative abundance of proteins in normal and diseased cells to each other. Previously, the study of protein expression patterns from cells has been confined mostly to the separation and analysis by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), which has been used extensively to study changes in protein expression in cell lines and bulk tissue specimens [Young and Tracy, 1995; Reymond et al., 1997; Franzen et al., 1995; Okuzawa et al., 1994]. Until now, it has been impossible to study protein expression profiles in pure microscopic cell populations of tissues which are responsible for the disease itself, are the target of the drug therapy, or are undergoing a toxic response to treatment. Protein fingerprints of disease at an early stage in defined cellular lesions could be of substantial clinical value for the development of strategies for early detection and/or treatment. Unfortunately, these histopathologically defined lesions represent subpopulations of cells comprising only a small fraction of the total number of cells present in a tissue section and may contain only hundreds of cells [Simone et al., 1998].

2D-PAGE analysis, although a powerful tool for the analysis of complex protein mixtures, is a laborious, time-intensive process not amenable to rapidly assessing changes in protein expression from hundreds or thousands of samples. This technique has limitations on its ability to analyze very small proteins (<7,000 Daltons), and would be unable to detect low or even moderately expressed proteins when analyzing lysates generated from the few hundreds of cells that comprise these important cellular lesions. Consequently, there is a need for technologies capable of sensitive, high-throughput protein display which would validate the correlation of phenotype with the presence or absence of a protein biomarker from small microscopic samples initially. Once the validation of the protein expression profile, consisting of a multiplex of hundreds of proteins, correlates with a particular phenotype a decision could be made to pursue one or more of the protein candidates in greater detail.

Surface-enhanced laser desorption ionization spectrometry (SELDI) has been recently described, and is reported with detection sensitivity in the attomole (10^{-18}) range [Hutchens and Yip, 1993; Kuwata et al., 1998a,b; Strauss, 1998]. This technology is an example of affinity-based laser desorption and ionization spectrometry where proteins of interest are directly applied to a biochip utilizing a defined small surface area (1–2 mm^2) coated with specific chemical "bait" matrices comprised of standard chromatographic supports (hydrophobic, cationic, anionic surfaces, etc.) or biochemical bait molecules such as purified proteins, ligands, receptors, antibodies, or DNA oligonucleotides [Hutchens and Yip, 1993; Kuwata et al., 1998a,b; Strauss, 1998; Brockman and Orlando, 1996]. The bound protein population can then be washed with a variety of washing buffers so that only those proteins that share common chemical characteristics are retained on the surface, and the subsequent mass map of all proteins retained is generated simultaneously. This analysis generates a protein "fingerprint" based on the combined precise molecular weight signatures of each individual protein bound and ionized off the specific bait surface employed.

We have extended this technology to construct a sensitive, reproducible protein biomarker protein display tool for the study of disease progression in discrete cell populations from actual human tissue as a model system for the rapid analysis and characterization of protein expression changes from microscopic-sized input material. Defined, patient-matched cellular populations, representing the spanning progression of malignancy, were analyzed using limited study sets of human solid tumors from five different tissues: esophageal, prostate, breast, ovary, and colon. To this end, laser capture microdissection (LCM) was employed to acquire histopathologically defined cell populations directly from stained human tissue sections [Emmert-Buck et al., 1996; Bonner et al., 1997] and cellular lysates from the captured cells were applied to the SELDI biochip surface for protein biomarker display.

MATERIALS AND METHODS

Tissue Preparation and Staining

Frozen section slides, 8 μm thick, were prepared from each case as previously described except AEBSF (Boehringer Mannheim, Germany) was added to the staining baths at a final concentration of 2 mM to inhibit proteases [Simone et al., 1998; Emmert-Buck et al., 1996;

Bonner et al., 1997]. Based on careful review of the histologic sections by a pathologist (JG), each microdissection was estimated to have >95% purity.

Laser Capture Microdissection

Stained tissue sections were subjected to LCM (Pixcell 100, Arcturus Engineering, Mountain View, CA) as previously described [Simone et al., 1998; Emmert-Buck et al., 1996; Bonner et al., 1997] except cells were lysed within 5 min of capture. Microdissected cells were lysed directly with 2 μl of an extraction buffer containing 1% (w/v) Triton-X-100 (Sigma, St. Louis, MO), 1% (w/v) MEGA 10 (ICN, Aurora, OH), and 1% (w/v) octyl-β-glucopyranoside (ESA, Chelmsford, MA), and 0.1% SDS (BioRad, Hercules, CA) in a standard 1X PBS.

SELDI Analysis

SELDI analysis was performed using an aliphatic reverse phase chip (H4 Protein Chip™, Ciphergen, Palo Alto, CA). The bait surfaces on the chip were pretreated with 2 μl of acetonitrile (Sigma). Shortly before the acetonitrile completely evaporated, 2 μl of the lysate was applied to the bait surface. The analyte was allowed to concentrate by air-drying followed by washing two times for 5 min in 1X PBS. Then 0.3 μl of a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Sigma), the energy-absorbing molecule of choice, was applied to the washed surface of the chip and allowed to crystallize.

RESULTS

Sensitivity and Reproducibility of Protein Biomarker Profiling from Defined Cellular Lesions

To assess the reproducibility of the molecular weight signatures generated by the SELDI protein fingerprint of LCM-derived cells, 1,500 cells of esophageal normal epithelium were microdissected, lysed, and applied to a predefined bait surface on a hydrophobic interaction C18 biochip. A dataset was generated using the cumulative detection of proteins from 12 of the possible 100 different addressable regions within a single biochip surface region. Each of these experiments was performed in triplicate (three separate cumulative groupings of 12 different areas of the same spot), giving a total of 36 data points for each protein peak analyzed. A representative "mass chromatogram" is shown at the top of Figure 1A, with eight different proteins chosen for further analysis. Different peaks and shoulders were chosen for their diversity in relative intensity to one another. Analysis was performed using normalization to protein #8, which appeared consistently in all experiments.

The reproducibility of the detection of the tissue proteins is shown in the middle of Figure 1A, with the relative proportionality of a subset of these peaks to one another assessed (bottom of 1A). The coefficients of variance and standard deviations of each of the relative intensity of these proteins and relative ratios between peaks reveal a high degree of reproducibility of the protein profile of the same sample regardless of which subregion of the biochip surface the laser targets.

The analysis of the reproducibility of the protein profile obtained from several independent applications of equivalent loadings of the same lysate was performed using a lysate of microdissected esophageal normal epithelium from the same case. In this study, the lysate from 30,000 cells from the same patient was applied to 20 different C18 hydrophobic interaction bait surfaces (for an average protein load of 1,500 cell equivalents per bait surface) and analyzed in triplicate (12 regions per dataset) for over 720 data points per protein analyzed (Fig. 1B). The coefficients of variance and standard deviations generated from these analysis reveals that the molecular mass fingerprint of a given lysate can be reproducibly attained from any independent application.

We tested the sensitivity by titrating lysates of microdissected normal esophageal epithelial cells from 1,500 to 0 cells/application (Fig, 1C). Each fingerprint was performed in triplicate, with a representation of one study set shown. The results of this experiment are represented as a gel-like display which takes the data from the mass chromatogram and presents the data as if one is looking at a standard 1D-SDS-PAGE gel "stained" for proteins, with the molecular weight ranges displayed at the same scale as that seen in the chromatogram. Normalization of the peaks of the gel display for direct comparison of each of the samples was performed using the signature of the applied sinapinic acid matrix. The number of proteins reproducibly detected in each of the matched triplicates for each cell number set decreases as the number of cells decrease. The lower limits of a reproducible protein fingerprint are in the 25–250 cell range.

When sensitivity is calculated as a product of cell equivalencies, the detection limits become even more dramatic (Fig. 1C). This calculation is based on the fact that each individual bait surface on the protein biochip contains approximately 8,000 theoretically addressable regions, based on the area of a circle. Each of the individual protein profiles generated in these and all datasets generated in this study represent the cumulative detection of 12 different regions within one sample spot, so that the sensitivity of detection in terms of cellular equivalents is $12/8{,}000^{th}$ or 0.15% of the total lysate of the cells applied to the surface within one spot. This means that the biomarker protein profile seen represents the lysate of two cell equivalents.

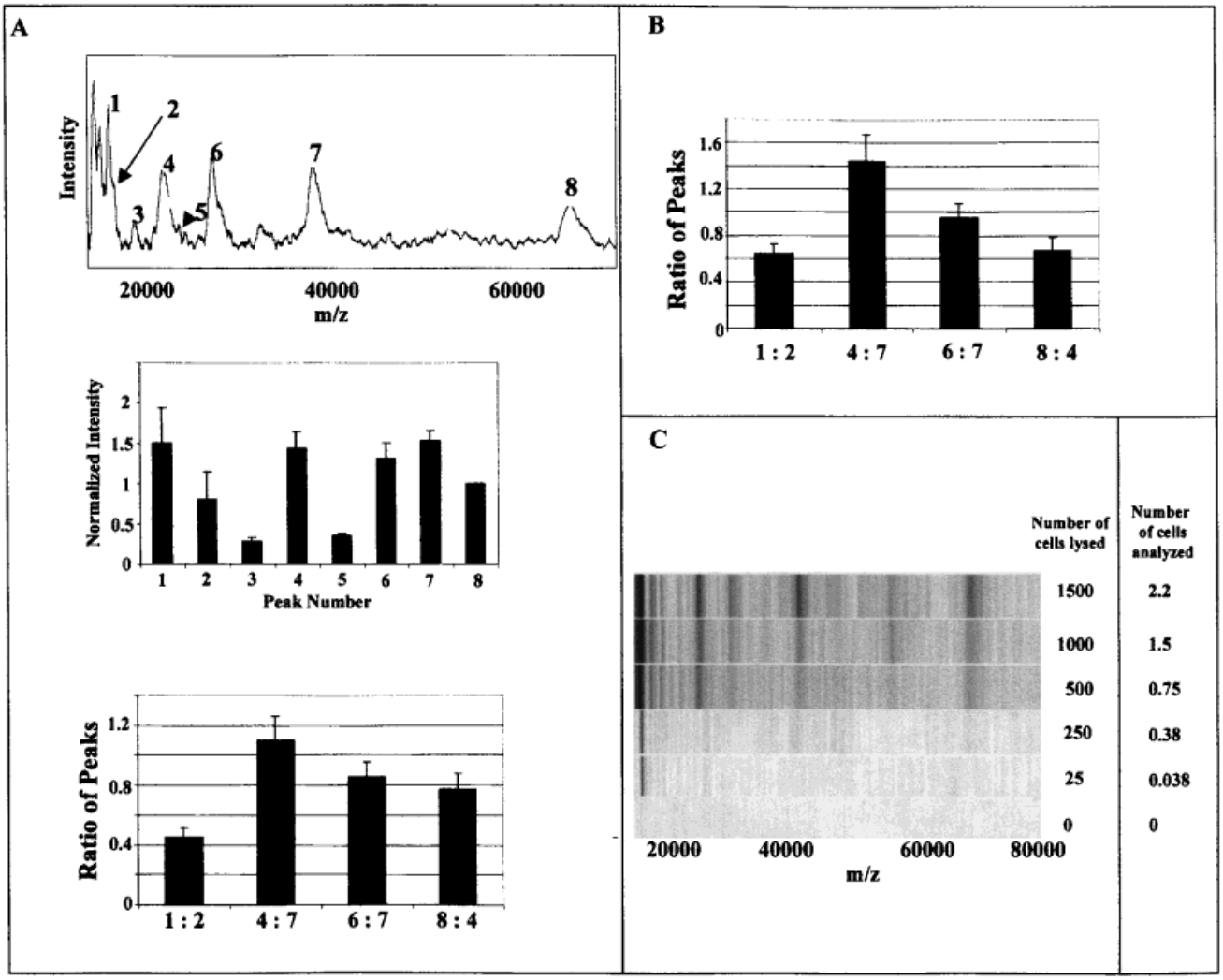

Fig. 1. SELDI protein profiles of LCM-derived cellular lysates are reproducible and sensitive. **A**: Reproducibility within a spot: 1,500 cells of esophageal normal epithelium were microdissected, lysed, and applied to a single spot on a SELDI hydrophobic interaction C18 surface chip and a dataset generated using the cumulative detection of proteins from 12 of the possible 100 different addressable regions within a single spot. The raw mass map for one of these regions is shown at the top, with the molecular weights displayed on the x-axis and relative intensity along the y-axis. The middle diagram represents the statistical analysis of the reproducibility of the relative intensity of each of the selected proteins to be detected in any of the 12 regions queried. Data is normalized to peak 8 (average C.V = 15.1%). Ratio analysis demonstrating the reproducibility of the relative intensity of one peak to the next is shown at the bottom of the panel (average C.V. = 12.9%). **B**: Reproducibility of the same lysate between spots. Lysate from 30,000 cells from the same patient were applied to 20 different spots (for an average protein load of 1,500 cell equivalents per spot) on a SELDI hydrophobic interaction C18 surface chip and analyzed in triplicate (12 regions per dataset) for over 720 data points per protein analyzed. The reproducibility of the ratios of the relative intensity of each selected peak to the other is shown (average C.V. = 11.9%). **C**: Fifteen separate microdissections of a decreasing number of cells were analyzed by SELDI protein fingerprinting (three microdissections for each cell number) and compared to a blank microdissecting cap. The gel-like display is shown as a representation of the direct alignment of each of the mass spectra to each other. A representation of one study set shown. Normalization of the peaks for the direct comparison of each of the samples was performed using the signature of the applied sinapinic acid matrix. Additionally, an advanced "peak identification" program, included in the SELDI software package, was applied to each of these samples (in triplicate), and the number of proteins whose signature was reproducibly 4 SD above noise was identified and are shown next to the gel view display. Direct cell equivalents were calculated in the following manner: Each of the individual protein profiles generated in these and all datasets generated in these studies represent the cumulative detection of 12 different regions within one sample spot, so that the sensitivity of detection in terms of cellular equivalents is 12/8,000th or 0.15% of the total lysate of 1,500, 1,000, etc., cells applied to the surface within one spot.

Rapid Protein Profiling of Microdissected Tumor and Normal Human Cancer Cells

A powerful application of this technology would be to directly protein fingerprint normal and diseased cells from the same patient and validate the clinical utility of a protein biomarker whose presence or absence correlates with disease. A study set of eight different esophageal cancer cases, which contained both tumor and normal epithelium, was chosen for extensive analysis. Three separate independent microdissections of eight different patients' tumors and normal esophageal epithelia were performed and the whole cell lysate subjected to SELDI analysis via the use of a hydrophobic interaction C18 binding surface. Each replicate was run in triplicate, giving a total of 72 data points for each protein peak analyzed. The results are shown in Figure 2. Two proteins were found to be present in a disease-specific manner, one of which was upregulated in a tumor-specific manner in seven out of eight cases analyzed, and one found to be specifically present in normal epithelium from all eight cases examined. The analysis for the protein fingerprint in the low mass region is shown in Figure 2A, the higher mass region in Figure 2B. Coefficients of variation and standard deviations for these analyses indicate that the relative abundance of these two proteins may serve as valid biomarkers that correlate with disease.

Protein Biomarker Profiling of Patient-Matched Metastatic vs. Nonmetastatic Human Cancer Cells

The ability to assess changes in protein expression occurring during tumor progression will aid in the elucidation of the fundamental mechanisms underlying metastasis in patients. To investigate the potential of SELDI analysis of LCM-derived cells to study this process, we analyzed

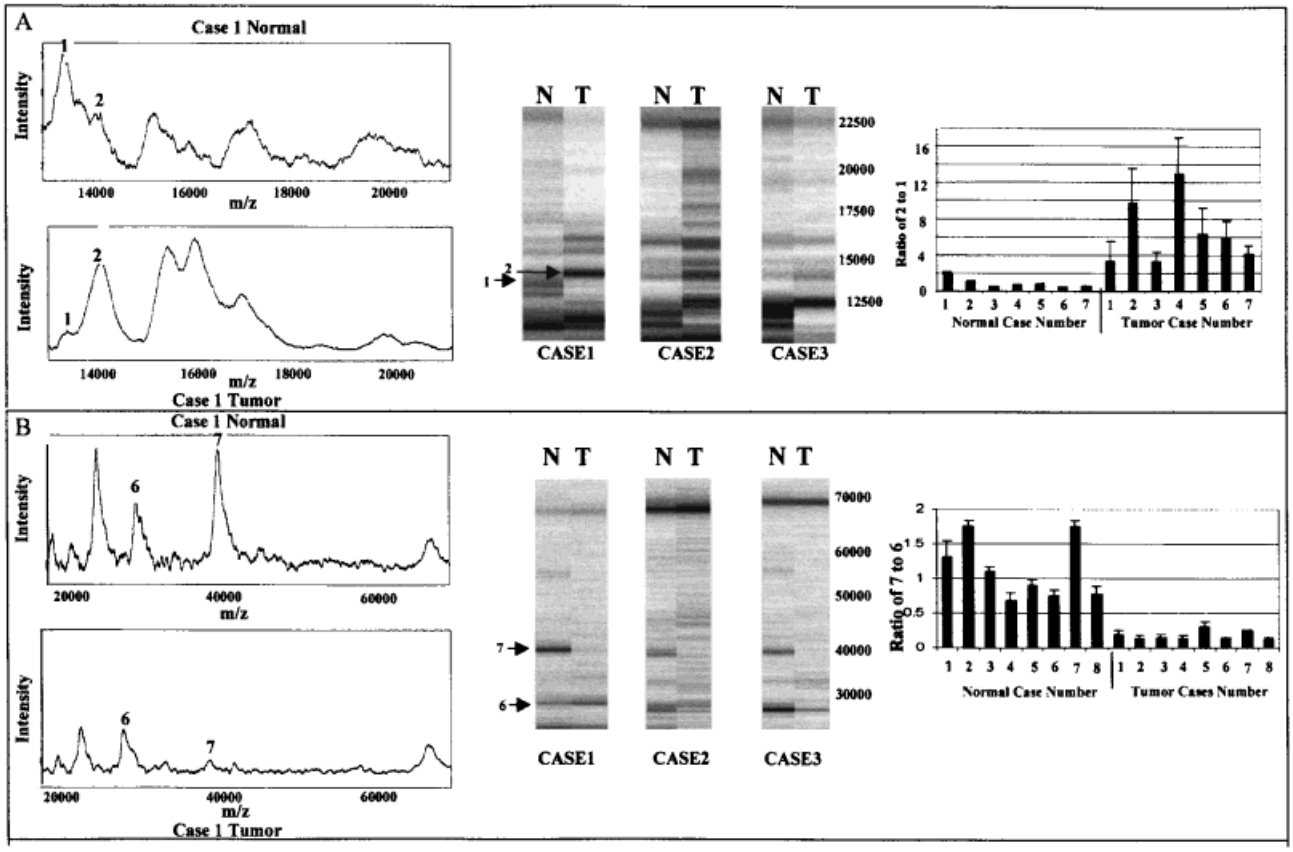

Fig. 2. SELDI analysis of microdissected esophageal epithelium reveals proteins disregulated in a disease-specific manner. **A,B**: Three separate microdissections of eight different patients' matched tumor and normal epithelial cell populations were subjected to SELDI analysis via the use of a hydrophobic interaction C18 binding surface. Each replicate was run in triplicate, giving a total of 72 data points for each protein peak analyzed. The analysis of the protein fingerprint in the low mass region is shown in **A**, the higher mass region in **B**. A representative mass map from one case (Case 1) is shown on the left side of each panel with the normal and tumor fingerprint shown (top and bottom, respectively) for each mass region. A gel-like representation is displayed for that particular case as well as the fingerprint for two other cases. Proteins 1, 2, 6, and 7 are labeled for orientation. All cases analyzed in the study set were then subjected to analysis as a ratio of relative intensity of the selected proteins to one another and the statistical results shown on the right side of each panel. **A**: Average C.V = 12.7%, 29.4% for the normal and tumor microdissections, respectively. **B**: Average C.V. = 10.5%, and 18.9% for the normal and tumor microdissections, respectively.

1,500 microdissected cells of patient-matched colon normal epithelium, primary cancer, and hepatic metastasis. The experiment was performed in triplicate, with the representation of one of the experiments shown. As a comparison, we analyzed the normal liver cells from the same hepatic metastasis tissue section, which were microdissected from the same case. The results (Fig. 3A,B) are shown in both the raw chromatographic map and corresponding gel image and reveal that a normal epithelial cell and invasive carcinoma specific fingerprint can be identified. Thus, in this individual patient the protein pattern profile was distinct from the primary colon epithelium.

Protein Fingerprinting of Early Disease Cellular Lesions in Human Tissue

A crucial aspect to the study of disease progression and the identification of protein changes that occur as the disease evolves is the ability to detect changes very early in the disease cycle. An example of a microscopic lesion that can be specifically analyzed for early changes is a premalignant cancer cell [Krizman et al., 1996]. Normal, premalignant prostatic intraepithelial neoplasia (PIN), and frankly invasive cancer cells were microdissected from one stained human tissue section and a protein biomarker fingerprint obtained (Fig. 4A). The mass map shown represents the protein profile from 1,500 cells acquired by LCM. Additionally, the corresponding patient-matched stromal cells (1,500 cells) were microdissected for analysis. A gel image of the raw mass data is presented in Figure 4B. All samples from this patient were run in triplicate, with the representation of one experiment shown. Two proteins having molecular weights of 28,000 and 32,000 Daltons were

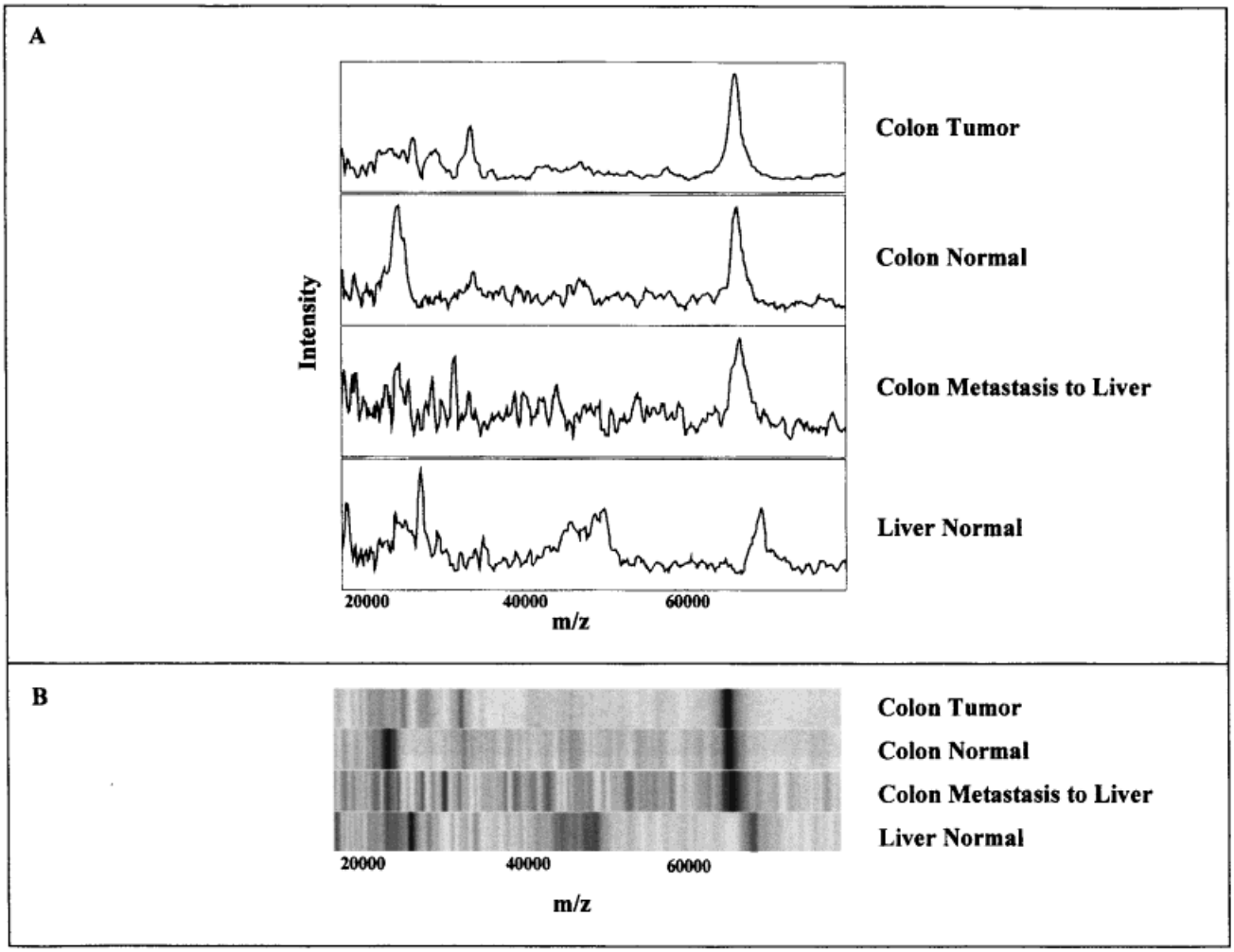

Fig. 3. SEDLI analysis of colon carcinogenesis. **A**: SELDI mass map of the lysates from 1,500 microdissected cells of colon normal epithelium, primary cancer, and hepatic metastasis from one patient. The experiment was performed in triplicate, with the representation of one of the experiments shown. As a comparison, we analyzed the normal liver cells, which were microdissected from the same case. **B**: A gel-like display is shown as a representation of the direct alignment of each of these four mass spectra (shown in **A**) to each other.

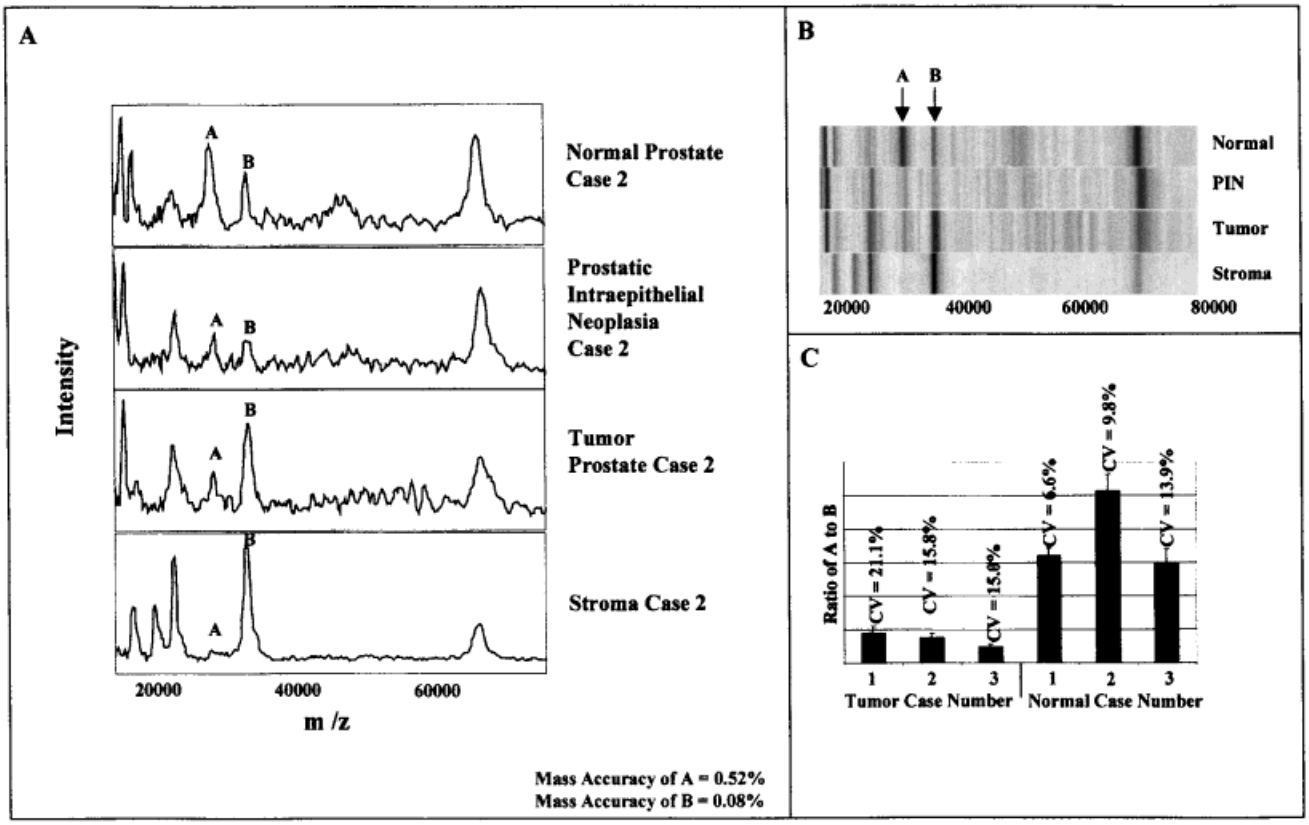

Fig. 4. SELDI analysis of prostate carcinogenesis. **A**: The mass map shown represents the profile from 1,500 normal, preinvasive neoplasia (PIN), and invasive carcinoma cells acquired by LCM from one case (Case 2). Additionally, the corresponding patient-matched stromal cells (1,500 cells) were also microdissected for analysis. **B**: A gel-like image of the raw mass data shown in **A**. All samples from this patient were run in triplicate, with the representation of one experiment shown. Two proteins, A and B, having molecular weights of 28,000 and 32,000 Daltons, respectively, were found to be reproducibly differentially expressed in this patient and are indicated in both **A** and **B**. **C**: Ratio analysis of **A** vs. **B** from an additional study set of two other (Cases 1 and 3) patient-matched tumor and normal prostatic epithelium (average C.V. = 17.2% and 10.1% for the tumor and normal microdissections, respectively).

found to be reproducibly differentially expressed in this patient and are indicated in Figures 4A,B. The results from this limited study set show that 1) each cell type has its own unique biomarker fingerprint, and 2) the PIN cell type seems to have qualities of protein expression that are intermediary between the normal and tumor cell types. The stromal population has its own reproducible fingerprint, and can be discriminated from all of the epithelial cell profiles regardless of disease stage. An additional study set of two other patient-matched tumor and normal prostatic epithelium was subjected to the same analysis as above (Cases 1 and 3). Specific, reproducible protein biomarker changes were seen in a disease-associated manner in all three cases analyzed. The relative ratio of these two proteins correlates with the disease state of the cell, and is reproducible (Fig. 4C). This protein profile could reliably be generated from pure precancerous human cell populations microdissected directly from tissue.

Discriminatory SEDLI Analysis of Different Tumor Types

A practical application for both clinical and research studies utilizing SELDI fingerprinting of LCM-acquired cell populations would be to develop diagnostic fingerprints that are disease- and organ-specific. To assess the feasibility of this concept, we tested the ability of SELDI to discriminate between tumor epithelium from breast, ovary, esophageal, and prostate tumors from two different patients from each tumor type.

Fifteen hundred cells from each case were microdissected and analyzed as described above and the resultant spectra are shown in Figure 5A,B. Two separate cases of each tumor type were microdissected and analyzed. Both a protein biomarker gel-like display and chromatographic map are shown for direct comparisons. Although a more extensive study set will be required to statistically define parameters for tumor-type specificity, each tumor type displays a protein profile that contain intriguingly unique general characteristics.

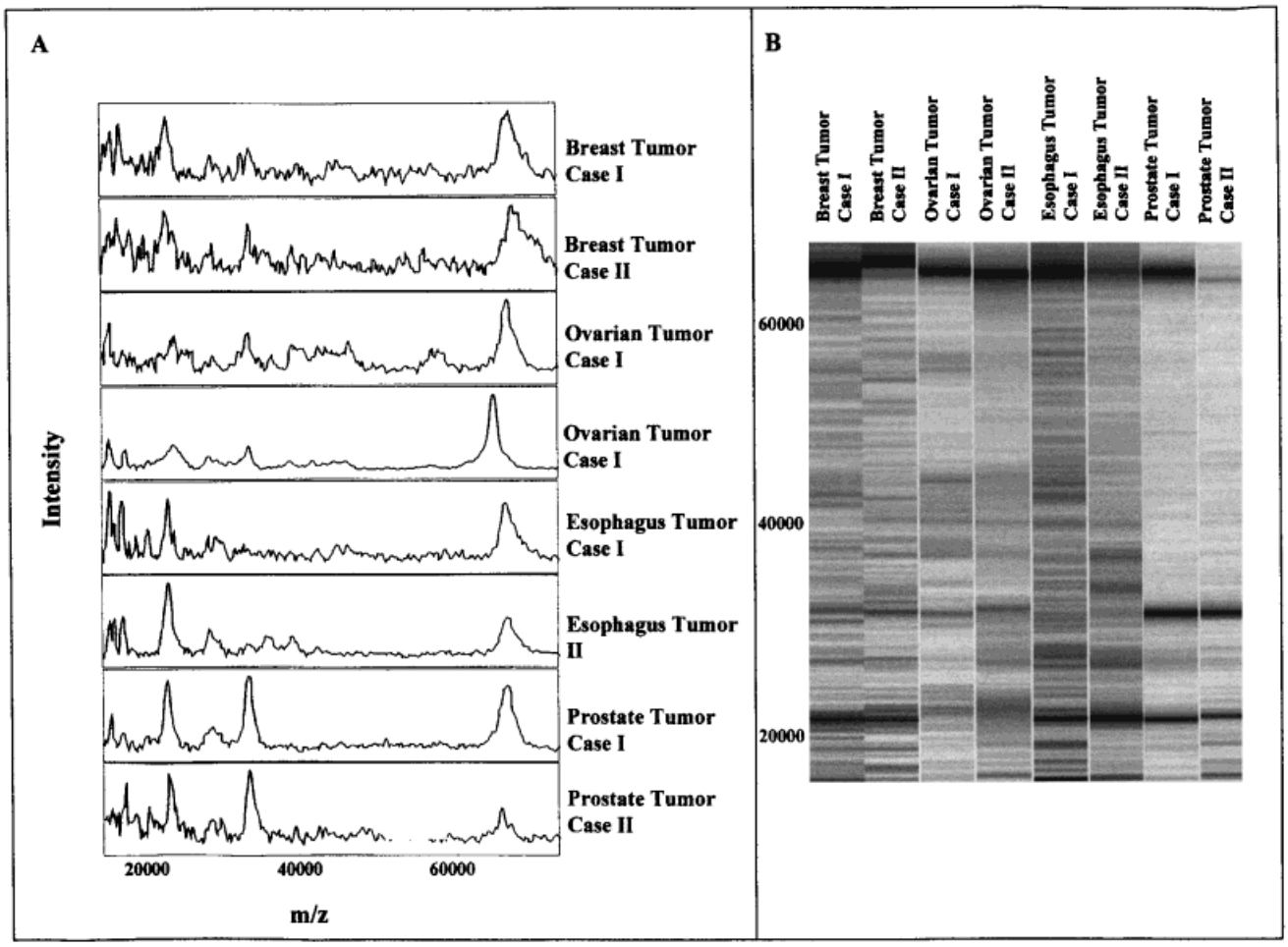

Fig. 5. SELDI protein profiles of different microdissected human solid tumor epithelium. **A**: 1,500 cells from each case were microdissected subjected to SELDI analysis and the resultant spectra are shown. Two different cases for each tumor type were analyzed. **B**: Gel-View™ representation of the data.

DISCUSSION

We conclude from our initial results that SELDI analysis of LCM-derived cells can be achieved with a high degree of reproducibility, resolvability, sensitivity, specificity, and discrimination. Results are obtained in less than 15 min from the performance of the microdissection to the generation of the protein fingerprint . These operating conditions allow for the exciting possibility that rapid protein biomarker fingerprinting analysis of cells from diseased cellular lesions directly from patient tissue is possible. The ability to rapidly monitor protein expression states could potentially add prognostic and diagnostic options to the clinical decision-making process, as well as establishing the exciting possibilities of the evaluation of therapeutic efficacy evaluation on the diseased cells themselves. These changes were reproducibly demonstrated within a tumor type from study sets of esophageal and prostatic cancer tissue specimens. Furthermore, for the first time we show the ability to study true disease progression in the context of LCM-acquisition of pure normal, premalignant, and invasive cancer epithelial cells from the same patient's tissue section and subsequent SELDI protein profiling.

Results presented in this study are generated from only one set of bait substrate and wash conditions. The protein fingerprints shown in the present study represent only a small portion of the entire cellular repertoire of proteins in the analyzed cells. Retentate Mapping™, devised at Ciphergen Biosystems, Inc., utilizes various protein capture surfaces and washing conditions to exploit the intrinsic binding characteristics of the proteins and generate a more expansive and detailed protein fingerprint. This approach greatly increases the number of proteins that can be characterized, because it would physically separate proteins into an array of classes (such as cationic/anionic or hydrophilic/hydrophobic).

This system will be applied to LCM-procured cellular lysates for a more defined disease progression analy-

sis, and is currently on-going. However, dramatic and reproducible changes in the cellular protein profile of patient-matched microdissected tumor and normal epithelium were seen even though a subpopulation of the full cellular protein complement was analyzed. The unique differences between tumors may illustrate the wide range of protein differences that can occur in epithelial cells from different solid tumor types. Novel insights into the protein expression patterns between different types of tumors could aid in the development of new treatment strategies, or in the employment of the best existing treatment option. Diagnosis and prognosis from the limiting amounts of cells generated by fine-needle aspirants or sentinel node analysis could be achieved very rapidly if tumor-specific or grade-specific profiles could be generated and used as a template.

In conclusion, a novel protein biochip was used to generate protein fingerprints from microscopic cell populations directly from human tissue. This technology attained a high degree of reproducibility, sensitivity, and specificity. Tissue protein profiling will be an essential component of pharmacoproteomics in the future. This will have a direct impact on therapeutic assessment, drug or surgical intervention strategies, toxicity monitoring, and disease diagnosis.

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