Integral Protein Microarrays for the Identification of Lung Cancer Antigens in Sera That Induce a Humoral Immune Response*§

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The identification of biomarkers (both molecules and profiles) in patient sera offers enormous interest for the diagnosis of cancers. In this context, the detection of antibodies to tumor cell autologous antigens possesses great potential. The humoral immune response represents a form of biological amplification of signals that are otherwise weak because of very low concentrations of antigen, especially in the early stages of cancers. Herein we present the use of integral microarrays spotted with tumor-derived proteins to investigate the antibody repertoire in the sera of lung cancer patients and controls. The use of two-dimensional liquid chromatography allowed us to separate proteins from the lung adenocarcinoma cell line A549 into 1760 fractions, which were printed in duplicate, along with various controls, onto nitrocellulose coated slides. The sensitivity and specificity of the microarrays to detect singular antibodies in fluids were first validated through the recognition of fractions containing a lung marker antigen by antibody probing. Twenty fractions were initially selected as highly reactive against the anti-PGP9.5 antibody, and subsequent mass spectrometry analyses confirmed the identity of PGP9.5 protein in four of them. As a result, the importance of neighboring fractions in microarray detection was revealed due to the spreading of proteins during the separation process. Next, the microarrays were individually incubated with 14 serum samples from patients with lung cancer patients, 14 sera from colon cancer patients, and 14 control sera from normal subjects. The reactivity of the selected fractions was analyzed, and the level of immunoglobulin bound to each fraction by each serum sample was quantified. Eight of the 20 fractions offered p values < 0.01 and were recognized by an average of four reacting patients, whereas no serum from normal individuals was positive for those fractions. Protein microarrays from tumor-derived fractions hold the diagnostic potential of uncovering antigens that induce an immune response in patients with certain types of cancers. Molecular & Cellular Proteomics 7:268–281, 2008.

Early detection of cancer represents one of the main interests of physicians and biologists in the postgenomic era, as many hopes for cancer cure lay on an effective diagnosis in the early phases of tumorigenesis (1). To date, however, few approaches have confirmed their validity and benefit for general use. There is a compelling need to elucidate the function of thousands of novel genes and to develop an improved understanding of biological processes. DNA microarrays have proven to be a powerful technology to allow the large scale analysis of gene expression at the RNA level (2). As the proteome is the most functional compartment encoded for in the genome, proteomics has become an essential tool for complementing knowledge derived from genomic analysis. There is a need to develop technologies for proteomics that allow the systematic analysis of thousands of proteins in parallel for basic biological research, to develop a better understanding of disease processes and identification of novel biomarkers and therapeutic targets (3).

In this context, much interest exists in identifying markers for the early detection of lung cancer. One way to perform this task is to detect cancer antigens that elicit a humoral response during tumorigenesis. There is substantial evidence for an antibody-mediated immune response to cancer in humans, as demonstrated by the identification of antibodies against a number of intracellular and surface antigens in serum from patients with different tumor types (4). Immunogenicity may depend on the level of expression, post-translational modification, or other types of protein processing that may be tumor specific. Therefore, immunogenicity may be best determined with proteins isolated from tumors or tumor-derived cell lines. In lung cancer, as in other tumor types, the majority of tumor-derived antigens that have been identified that elicit a humoral response are not the products of mutated genes. These antigens include differentiation antigens and other proteins that are overexpressed in tumors, such as the oncogenic proteins L-Myc and C-Myc, which have been found to elicit autoantibodies in some patients (5). Immunogenic proteins also include cancer/testis antigens, which are normally expressed only in the human germ line but are also expressed in various tumor types, including lung (6). In lung cancer, autoantibodies have been demonstrated against certain proteins, including the P/Q type voltage-gated calcium channel, annexin I and II, protein gene product 9.5 (PGP9.5)1 (7),

1 The abbreviations used are: PGP9.5, protein gene product 9.5; PBS, phosphate-buffered saline; RPLC, reversed-phase liquid chromatography; UCH-L1, ubiquitin carboxyl-terminal hydrolase isozyme 1;
livin and survivin, peroxiredoxin-I, NY-ESO-1. There is some evidence that the occurrence of autoantibodies to specific antigens in lung cancer may have prognostic relevance (8, 9). Remarkably, tumor regression has been demonstrated in some patients with small cell lung carcinoma and autoantibodies to onconeural antigens (10).

PGP9.5 (ubiquitin COOH-terminal esterase L1, or UCH-L1) is an ubiquitin COOH-terminal hydrolase that was previously identified as an antigen that induces an antibody response in lung cancer (7). PGP9.5 is widely expressed in neuronal tissues at all stages of neuronal differentiation and may be expressed during neuroendocrine differentiation of lung cancer. Ubiquitination and targeting of cellular proteins for subsequent degradation via ubiquitin-mediated proteolysis are an important mechanism regulating a broad spectrum of cellular processes. In tumors, increased deubiquitination of cyclins by PGP9.5 may contribute to the uncontrolled growth of somatic cells (11, 12).

Proteomics approaches as a method of identification of biomarkers in cancer samples hold the promise to assist in individualizing the diagnosis (13). Several reviews have reported on applications of protein microarrays during the last years (14–17). And a number of research papers have implemented protein microarray platforms for the analysis of autoimmune reactivity. Robinson et al. arrayed 196 known proteins, each representing major antigens targeted by autoantibodies, for studies of autoimmune disease (18). Mintz et al. developed a screening method based on phage display to select peptides recognized by circulating tumor-associated antibodies (19). A microarray containing 15 autoantigens for the detection of autoantibodies in rheumatoid autoimmune diseases was created by Feng et al. (20). To date, the majority of studies of antigen-antibody reactivity using microarrays have relied on recombinant proteins or synthetic peptides as arrayed features (although with the first examples of cell-free self-assembled protein microarrays recently published, an alternative way is open; (21)). However, using recombinant proteins as printed antigens may elicit false results due to the lack of proper post-translational modifications (22). A lack of representation of specific post-translational modifications that occur in the target tissues, limits the repertoire of antigens that may be identified.

We have implemented a novel protein microarray based approach for the identification of tumor antigens that elicit autoantibodies in colon cancer. The strategy of using liquid-based multidimensional procedures to separate proteins in lysates prepared from tissues or cell lines, allows fractions containing distinct proteins to be arrayed and interrogated using various types of probes (23). To this end, we have used a liquid-based two-dimensional fractionation system to separate and array the whole content of proteins expressed in a lung adenocarcinoma cell line. The so-constructed integral protein microarrays were hybridized with PGP9.5 antibody preparations, and with sera from lung cancer patients, to determine if some fractions contained PGP9.5 protein that reacted specifically with antibodies in lung cancer sera (Fig. 1). We further validated the expression of PGP9.5 in those fractions and the specificity of the immune response directed against it in lung cancer. In this study, we conclude the utility of this approach to identify proteins that elicit a humoral response, as well as the detection of PGP9.5 autoantibodies in serum of patients with lung cancer. Our findings indicate the potential of protein-based microarrays to delineate the repertoire of tumor-specific autoantibodies detectable in serum of cancer patients.

**EXPERIMENTAL PROCEDURES**

**Serum Samples**—Following informed consent, sera were obtained at the time of diagnosis from 54 individuals: 18 patients with adenocarcinoma lung cancer, 18 patients with colon cancer, and 18 non-

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HPLC, high performance liquid chromatography; ESI, electrospray ionization; Q-TOF, quadrupole time-of-flight.
disease normal subjects. Cancer patients and normal subjects were gender and age matched. All of them were assayed with trial versions of the microarrays and finally, 14 sera of every type were selected for the definitive studies.

**Cell Culture and Cell Lysate Preparation**—A549 lung adenocarcinoma cells were cultured (6% CO₂, 37 °C) in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml), all purchased from Invitrogen. The cells were solubilized in lysis buffer (24) containing 6 mM urea (ICN Biomedicals, Inc.), 2 M thiourea (Sigma), 1.0% n-octyl β-D-glucopyranoside (OG, Sigma), 2 mM ditherythritol (Sigma), protease inhibitor mixture (Boehringer), and 2% ampholytes, pH 3.5–10 (Bio-Rad). Lysates were collected, RNase A (10 U/ml) and DNase I (100 U/ml) were added, and the solution left on ice for 30 min. The supernatant was vortexed for 5 s, clarified by centrifugation (20,000 x g for 10 min), and collected.

**Protein Fractionation**—A preparative-scale Rotofor (Bio-Rad) was used to separate proteins in the first dimension (25). Whole cell protein extracts were diluted to 55 ml with running buffer (the same buffer as the lysis buffer except that 0.5% OG was used instead of 1.0%), and separated by isoelectric focusing for 6 h (10 °C). 20 separate fractions were collected. The protein concentration and pH of each fraction was measured as previously described (25).

The high performance liquid chromatography (HPLC) reversed phase column R2/H (Applied Biosystems) was used for the separation of proteins in the second dimension. Separations were performed at a flow rate of 1.3 ml/min using water/acetonitrile gradients (solvent A: 98% H₂O, 2% acetonitrile, 0.1% TFA; solvent B: 90% acetonitrile, 10% H₂O, 0.1% TFA). The gradient profile used was as follows: (0) 95% solvent A for 2.5 min; (1) 95 to 75% A in 2.5 min; (2) 75 to 35% A in 40 min; (3) 35% A for 5 min; (4) 35 to 15% A in 5 min; (5) 15 to 5% A in 5 min; (6) 5 to 95% in 5 min. Protein fractions were collected every 30 s (88 fractions from each 1D fraction) starting 1 min into the gradient, then immediately frozen at –80 °C. The fractions were lyophilized under vacuum, and resuspended in 25 µl of 60% phosphate-buffered saline (PBS), 40% glycerol.

**Protein Microarrays**—A total of 3872 features were prepared that consisted of 1760 distinct A549 fractions in duplicate, 52 positive, and 300 negative controls, and arrayed onto nitrocellulose membranes supported on glass slides (Schleicher and Schuell) using a 32-pin Flexys arrayer, as previously described (25). Biotinylated BSA was printed to act as a “landmark” to orient the arrays.

**PGP9.5 Detection**—Microarray Hybridization—Primary anti-PGP9.5 rabbit polyclonal antibody (Biogenesis) was applied to each microarray in 100 µl blocking solution (PBS containing 3% non-fat dry milk), normally diluted at a concentration of 1:100, for 1 h. The slides were washed five times in PBST (PBS, 0.1% Tween-20) for 1 min, followed by another three 1 min cycles of washing in PBS. Biotinylated anti-rabbit IgG (GE Healthcare) was applied to the hybridization chamber at a dilution of 1:50 in blocking solution. Following 1 h incubation, the membranes were washed in PBST four times for 1 min and twice in PBS for 1 min. Streptavidin, R-phycocerythrin (Molecular Probes) was added at a dilution 1:100 for 20 min. The slides were washed four times in PBST for 1 min, two times in PBS for 1 min, and then centrifuged at 200 x g to dryness. The microarrays were imaged at 550 nm using a GeneTAC LS-IV scanner (Genomic Solutions).

Patient serum was analyzed with the microarrays. Each slide was placed in its hybridization chamber inside a GeneTAC Hybridization Station (Genomic Solutions). A volume of 100 µl serum was added at a 1:50 dilution in blocking solution as a source of primary antibody, and allowed to hybridize for 2 h at 22 °C under agitation. The microarrays were washed four times in PBST for 1 min, followed by another two 1 min-cycles of washing in PBS. Secondary antibody reaction with a biotinylated anti-human IgG (GE Healthcare) and fluorescent detection were performed as described in the previous paragraph.

**Analysis of Protein Microarray Images**—Scanned microarrays were analyzed using the GeneTAC Biochip Analyzer software package (Genomic Solutions). Images were manually fitted with a grid and the spot intensities were measured. The local background was subtracted from the signal at each spot, and the resulting average intensity of each spot pixel determined. Spots and/or areas with obvious defects such as signal lower than background or high background were excluded from subsequent analysis.

**Mathematical and Statistical Analysis of Antibody Reactivity**—Variable signal brightness between slides was adjusted by dividing from each average intensity value the median spot intensity measures within each patch (a rectangular area of dots printed by the same pin). Resulting intensity measures less than 10 were set to 10. Differences between batches of slides that were printed and hybridized as groups were observable in the data, so that we compared the patch-adjusted intensity values to the median of the values for normal samples. Normalized data were subjected to different analyses: ratio of intensities for PGP9.5 antibody versus normal sera; one-sided χ² tests comparing anti-PGP9.5 antibody versus sera, and lung versus normal sera; selectivity for lung cancer sera; and classification of sera type according to a subset of fractions. As protein spreading across contiguous fractions occurs during the separation process, neighboring fractions were taken into account for the analysis of each fraction. Consequently, a new mathematical algorithm was devised: an oval-like, fraction-centered area was determined for each fraction, ranging 2 more fractions both ways in the first separation, and 3 more fractions upwards and downwards in the second one (summing a total of 19 fractions per area), and weighting each fraction proportionally, according to the chromatographic distribution pattern for a given protein (supplemental Fig. 1). We categorized fractions as positively reacting if their relative reactivity met a series of stringent cumulative criteria: the ratio of the intensities for the average PGP9.5 antibody response as compared with the average normal sera response should be >100; the base-2 logarithm ratio of the adjusted intensity for antibody (or serum) with respect to normals should be at least 1.5, with a p value < 0.05; differential reactivity against cancer/non-cancer sera (a serum was termed “positive” in its reactivity when the adjusted value of its intensity was at least 1.75-fold the mean of the normals); specificity of the interaction for lung patient sera (as opposed to both lung and colon sera, or just colon); and reproducibility in the response for both duplicate spots.

**Protein Identification by Mass Spectrometry**—Two-dimensional reversed-phase liquid chromatography (RPLC) fractions were solubilized (1:1 PBS and NH₄HCO₃) and then subjected to trypsin digestion at 37 °C for 18 h. Protein identifications were performed by nano-flow capillary LC electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) tandem mass spectrometry (MS/MS) using a Q-TOF Micro (Micromass). ESI MS/MS tandem spectra were recorded in the automated MS to MS/MS switching mode, with m/z-dependent set of collision offset values. Doubly and triply charged ions were selected and fragmented using argon as the collision gas. The acquired spectra were processed and searched against the non-redundant Swiss-Prot protein sequence database using ProteinLynx Global Server (www.micromass.co.uk).

**Two-dimensional Polyacrylamide Gel Electrophoresis and Western Blotting**—The procedure followed was as described previously (26). Proteins were run in the first dimension by IEF. For the second dimension separation, a gradient of 11-14% acrylamide (Crescent Chemical) was used. Proteins were transferred to an Immobilon-P PVDF membrane (Millipore) or visualized by silver staining of the gels. The membranes were incubated with sera at a 1:200 dilution, and were then incubated with horseradish peroxidase-conjugated IgG antibodies (GE Healthcare) at a dilution of 1:100. Immunodetection
was accomplished by ECL. Patterns visualized were compared directly with Coomassie blue-stained blots from the same sample to determine correlation with protein patterns.

**RESULTS**

Isolation and Identification of PGP9.5 Antigen by Multi-D Liquid Chromatography Fractionation Strategy—Preparative quantities (~500 mg) of solubilized proteins isolated from the A549 lung adenocarcinoma cell line were resolved by Rotofor isoelectric focusing in the first dimension (25). Following a 6-h isoelectric focusing separation period, 20 fractions covering the pl range of 3.5–10 were collected in polypropylene tubes by vacuum harvesting. Each Rotofor fraction was separated in the second dimension by reverse-phase liquid chromatography into 88 fractions, for a total of 1760 fractions (Fig. 2). All fractions were lyophilized to dryness, resuspended in 25 μl PBS/glycerol, and aliquots of them were then used to prepare protein microarrays, one-dimensional and two-dimensional gels, and perform MS/MS analysis. The designation “xxyy” is used in this paper to represent the “yy” fraction of the RP-HPLC separation of the “xx” Rotofor fraction.

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**Fig. 2. Fractionation scheme.**

a, determination of amount of protein, pH, and protein concentration for Rotofor fractions. The concentration is indicated in the line above the bars and is expressed in milligrams per milliliter. b, RP-HPLC chromatogram from Rotofor fractions 8, 9, 10 of A549 cell lysate. c, two-dimensional display of the separation results for an A549 cell lysate. Twenty fractions from the first dimension are plotted as columns of squares, each of them representing a 3 s period in the second dimension. The intensities of the chromatograms are not scaled, so that the relative amount of protein can be inferred from the display. This is suggested by the color of the squares, ranging from light gray (minimum amount of protein) to black (highest peaks).
Autoantibody Detection in Lung Cancer Using Protein Arrays

(a) 6 7 8 25 kDa

(b) pl

(c) 0640 PGP9.5 25 kDa

(d) 1 160 kDa 28 MW 17 8

P1 P2 P3
Rotofor fractions were further separated by one-dimensional SDS-PAGE and Western blots were then performed with a polyclonal anti-PGP9.5 antibody (Fig. 3a). A unique band with an estimated molecular mass of 25 kDa was clearly visible in three consecutive fractions (6, 7, and 8), whose pHs ranged from 4.5 to 6.0. Two-dimensional PAGE analyses were performed on those fractions and their patterns matched against a previously annotated A549 master gel. In all cases, three distinctive spots were located in the same region where previous reports found three PGP9.5 variants (Fig. 3b). In the same way, two-dimensional RPLC fractions from those three Rotofor fractions were also separated by one-dimensional SDS-PAGE. The small number of them (two or three per one-dimensional fraction) that exhibited reactivity against the PGP9.5 antibody suggested a high efficiency in the separation process (Fig. 3c). The distribution of reactive proteins/antigens in different fractions was determined to allow comparisons of reactivities by Western blot and by microarray analysis. Finally, a close glance at those specific two-dimensional fractions by two-dimensional PAGE analysis confirmed the occurrence of the same three spots that matched the PGP9.5 variants, with an estimated molecular mass of 25 kDa and with a pl between 5.0 and 5.6 (Fig. 3d).

Protein Microarray-based Detection of Fractions Containing PGP9.5 Antigen by Antibody Probing — The volume of sample that every pin in the robot head can deliver is \( \sim 0.5 \) nL. Considering that the average concentration of total protein in each fraction was \( \sim 2.3 \) \( \mu \)g/\( \mu \)L and given that each reverse-phase fraction contained 1–10 proteins as identified by MS, our estimation of the amount of individual proteins in each dot would be \( \sim 200 \) pg with a wide range. We arrayed 3872 features on each slide, representing 1760 separate protein fractions in duplicate, from the A549 lung adenocarcinoma cell line, as well as positive, negative, and landmark controls. Slides were utilized for hybridization with anti-PGP9.5 polyclonal antibody and the scanned image of the slide showed that only certain fractions reacted against the specific antibody (Fig. 4a-c). The intensity of the fluorescent signal varied in an inter-dots fashion, reflecting likewise the different proportion of the specific detected protein, in relation to the total amount of protein in each fraction. The analysis showed a high signal to noise ratio, which provides excellent sensitivity and reproducible detection. The findings were also concordant with dot-blot controls for each fraction, run in parallel and developed with the ECL technology (results not shown). Scanned images were quantitatively analyzed for intensity of hybridization of spotted individual fractions with the antibody.

Control spots on the microarrays, including PGP9.5 antigen, PGP9.5 antibody, tetanus toxoid, rabbit IgG, and biotinylated control proteins, repeatedly showed similar reactivity in all assays. Slides from the same batch were hybridized in parallel with sera from cancer patients, as well as sera from control normal individuals. Initially, a set of 15 fractions exhibited greater reactivity with PGP9.5 antibody relative to normal sera (PGP9.5/sera ratio > 100) (Table I). Ten of those fractions revealed correspondence with fractions that were reactive by Western blot analysis (they came from positive one-dimensional fractions, nos. 6, 7, and 8, as seen in Fig. 3a), and all the remaining fractions but one corresponded to a neighboring one-dimensional fractions (four fractions came from no. 9). With respect to the separation in the second dimension, most of the fractions were eluted in the same range of acetonitrile percentage, from 58% to 54%. From the 15 fractions, we discarded one for further analysis: fraction 0139 was detected in an isolated location, in a low-pH Rotofor fraction, where separation does not perform so precisely as in the medium pH range, so this fraction was probably not well resolved. Consequently, we omitted it for the statistical studies. A deeper analysis focused in that region, which comprised 550 spots, revealed 20 fractions with \( p \) values < 0.05 and PGP9.5/sera fold change > 1.5. Fourteen of them fell into the more restricted area of pH range and acetonitrile content previously marked as being of interest, and are listed in Table I (columns third and fourth). Nine of them reflected exactly the same selection as with the PGP/sera ratio > 100 criterion, and another five were new. In addition, two of them had also proven to be positive by immunoblotting with the anti-PGP9.5 antibody (Fig. 3c).

Identification of PGP9.5 Antigen in Selected Fractions by Mass Spectrometry — For the 19 fractions that demonstrated greater reactivity with PGP9.5 antibody, we prepared a tryptic digest of the protein constituents of the fractions, which were subjected to identification by MS/MS (ESI-Q-TOF). Analysis of

**Fig. 3. Detection of the PGP9.5 antigen in the liquid fractions.** a. Western blotting analysis of lung adenocarcinoma A549 protein fractions. Twenty protein samples from the Rotofor fractionation were run in a one-dimensional SDS gel and their blots were tested against PGP9.5 antibody. Three consecutive fractions exhibited a band of an estimated molecular mass of 25 kDa. b, two-dimensional PAGE analysis of A549 lung adenocarcinoma cell proteins. Panel 1 shows A549 whole cell extract two-dimensional protein pattern after silver staining. Panels 2, 3, and 4 depict two-dimensional patterns of fractions no. 6, 7, and 8 from the Rotofor fractionation. In all four cases, the arrows point to PGP9.5 protein (form 1). That spot was taken as a reference to which all the panels were aligned (dotted line), in order to compare protein distribution across close pH ranges. c. Western blotting analysis of two-dimensional fractions from the RPLC separation. Fifteen fractions that were selected as highly reactive against anti-PGP9.5 antibody by protein microarray analysis were run in a one-dimensional SDS gel and hybridized with the same antibody. Fractions 0640 and 0739 reacted positively. Their subsequent content analysis by MS/MS revealed that UCH-L1 (PGP9.5 protein) was one of their component proteins. d, two-dimensional PAGE analysis of fraction 0739. The right-hand panel shows a close-up of a region with three spots (estimated MW of 25 kDa, pl range of 5.3–6.0) that matched the location of the three known PGP9.5 variants (P1–P3).
fractions 0640, 0739, 0841, and 0939 revealed that they contained the UCH-L1 protein (Fig. 4d-g). Significantly, the two fractions that exhibited substantially higher fold change values for anti-PGP9.5 antibody reactivity (fractions 0739 and 0640) were confirmed as true “PGP9.5 containing” fractions. With respect to fraction 0739 in the figure, for instance, the precursor ions m/z 605.9966 and 614.9975 resulting from the tryptic digest respectively matched with a 15- and a 17-amino acid sequence of UCH-L1 against protein sequence database with good accuracy (error = 0.06 and 0.07 Da). Identification of UCH-L1 as an element of this fraction was of interest as we previously identified the same protein isozyme as an antigen that induced a humoral response in lung cancer (7).

Protein Microarray-based Assay for Autoantibodies in Sera from Patients with Lung Cancer—Fourteen sera from lung cancer patients, 14 sera from colon cancer patients, and 14 control sera from normal subjects were individually hybridized to the A549 protein microarray (Fig. 5a and b). The reactivity of
TABLE I
List of 20 selected fractions and their reactivity against patient sera

Fifteen fractions were initially selected as highly reactive against the anti-PGP9.5 antibody; the selection was based on their greater reactivity against the antibody relative to sera (PGP9.5/normal ratio > 100, second column). A focused analysis in that region that considered only a subset of fractions, added five more cases to the list of candidates; those fractions exhibited p values < 0.05 (third column) and fold changes > 1.5 (fourth column). In addition, a subsequent MS/MS analysis (results summarized in the fifth column) probed that 4 of those 20 fractions did contain the PGP9.5 antigen as a constituent protein (UCH-L1). The next four columns depict the reactivity of the selected fractions when the microarray was incubated with 14 sera from lung cancer patients compared with 14 samples from colon cancer, and 14 normal sera.

| Fraction name | PGP/normal ratio (minimum 100) | p (PGP vs normal) (p < 0.05) | Fold change (PGP vs normal) (FC > 1.5) | p (lung vs. normal) (p < 0.05) | MS-MS | No. positive lung sera patients (of 14 patients) | No. positive colon sera patients (of 14 patients) | No. positive normal sera patients (of 14 patients) | One-dimensional fraction no. | Two-dimensional fraction no. | pH | % acetonitrile |
|---------------|-------------------------------|-----------------------------|-------------------------------------|--------------------------------|-------|----------------------------------|------------------------------------------------|--------------------------------|-----------------------------|-----------------------------|-------|----------------|
| 0139          | 218.71                        | NS                          | NS                                  | 0.0334                         |       | 3                                | 1                                              | 0                                           | 1                           | 39                            | 0.0–2.0 | 56.5–56.0          |
| 0637          | 120.37                        | 1.789E-03                   | 1.899                               | 0.0154                         |       | 4                                | 3                                              | 0                                           | 6                           | 37                            | 4.6–5.0 | 57.5–57.0          |
| 0639          | 399.80                        | 8.663E-05                   | 5.943                               | 0.0154                         |       | 4                                | 4                                              | 0                                           | 6                           | 39                            | 4.6–5.0 | 56.5–56.0          |
| 0640          | 405.19                        | 1.136E-03                   | 23.789                              | 0.0011                         | P09936| 7                                | 5                                              | 0                                           | 6                           | 40                            | 4.6–5.0 | 56.0–55.5          |
| 0641          | 269.09                        | 1.299E-04                   | 9.433                               | 0.0334                         |       | 3                                | 4                                              | 0                                           | 6                           | 41                            | 4.6–5.0 | 55.5–55.0          |
| 0733          | NS                            | 1.376E-04                   | 3.173                               | 0.0154                         |       | 4                                | 5                                              | 0                                           | 7                           | 33                            | 5.1–5.5 | 59.5–59.0          |
| 0734          | NS                            | 2.128E-03                   | 2.555                               | 0.0334                         |       | 3                                | 3                                              | 0                                           | 7                           | 34                            | 5.1–5.5 | 59.0–58.5          |
| 0739          | NS                            | 3.853E-05                   | 34.922                              | 0.0334                         | P09936| 3                                | 2                                              | 0                                           | 7                           | 39                            | 5.1–5.5 | 56.5–56.0          |
| 0740          | NS                            | 5.441E-04                   | 5.390                               | 0.0154                         |       | 1                                | 4                                              | 0                                           | 7                           | 40                            | 5.1–5.5 | 56.0–55.5          |
| 0741          | 156.67                        | 1.491E-03                   | 1.687                               | 0.0334                         |       | 3                                | 1                                              | 0                                           | 7                           | 41                            | 5.1–5.5 | 55.5–55.0          |
| 0742          | 340.13                        | NS                            | NS                                  | 0.0334                         |       | 3                                | 1                                              | 0                                           | 7                           | 42                            | 5.1–5.5 | 55.0–54.5          |
| 0839          | 102.00                        | NS                            | NS                                  | 0.0068                         |       | 5                                | 6                                              | 0                                           | 8                           | 39                            | 5.6–6.0 | 56.5–56.0          |
| 0840          | 214.24                        | 5.868E-03                   | 1.719                               | 0.0029                         |       | 6                                | 6                                              | 0                                           | 8                           | 40                            | 5.6–6.0 | 56.0–55.5          |
| 0841          | 392.61                        | 6.235E-05                   | 4.586                              | 0.0334                         | P09936| 3                                | 3                                              | 0                                           | 8                           | 41                            | 5.6–6.0 | 55.5–55.0          |
| 0842          | 301.68                        | 4.643E-05                   | 3.333                               | 0.0154                         |       | 4                                | 2                                              | 0                                           | 8                           | 42                            | 5.6–6.0 | 55.0–54.5          |
| 0843          | NS                            | 5.750E-03                   | 1.548                               | 0.0068                         |       | 5                                | 3                                              | 0                                           | 8                           | 43                            | 5.6–6.0 | 54.5–54.0          |
| 0939          | 214.50                        | 4.802E-04                   | 3.258                              | 0.0154                         | P09936| 4                                | 2                                              | 0                                           | 9                           | 39                            | 6.1–6.5 | 56.5–56.0          |
| 0940          | 229.60                        | NS                            | NS                                  | 0.0334                         |       | 3                                | 1                                              | 0                                           | 9                           | 40                            | 6.1–6.5 | 56.0–55.5          |
| 0941          | 292.94                        | NS                            | NS                                  | 0.0334                         |       | 3                                | 1                                              | 0                                           | 9                           | 41                            | 6.1–6.5 | 55.5–55.0          |
| 0942          | 123.73                        | NS                            | NS                                  | 0.1543                         |       | 1                                | 1                                              | 0                                           | 9                           | 42                            | 6.1–6.5 | 55.0–54.5          |

NS, nonsignificant fractions according to the criteria stipulated in the headings; P09936, UBL1 protein, ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1); -, UBL1 not detected.
the selected fractions was analyzed, in terms of their specificity for lung sera as opposed to normals. Results are summarized in Table I (columns sixth to ninth). Eight of the 19 fractions offered $p$ values $<0.01$, and only 5 showed nonsignificant values when incubated against the lung sera. Those patients for whom the adjusted intensity values were at least 1.75 greater than the signal from normal subjects were considered positive for that specific fraction. For almost all the cases analyzed, the number of lung positive patients ranged between 3 and 7, with an average of 4 reacting patients per fraction. For colon cancer patients, the reactivity figures were lower on average, with 3 positive patients per fraction, while for normal sera there were no patients showing response against any of those fractions. In the same sense, the adjusted intensity values for those fractions were higher for microarrays hybridized with sera from lung cancer patients than with both colon and normal sera.

Immunoblotting assays of some of the selected fractions were performed with sera from lung cancer patients, in order to validate the microarray findings. Fig. 5c gathers the images corresponding to two fractions when incubated with three different sera. In all cases, a band of around 25 kDa was distinctly visible in the blots, suggesting that a protein of such an estimated molecular mass in the fractions was immunoreacting against a specific antibody in the sera.

A global picture of the specificity for lung patient sera of
those and other fractions in the protein microarray format can be obtained from what we call a “digital Western blot.” This is a computerized image, generated with the processed data for every single interaction between the 3872 spots and the 14 lung tumor sera, relative to the 14 normal individual sera (Fig. 5d). It is essentially a heat map of the intensity data for all fractions using the TreeView presentation tool. However, the fractions are arranged in a way to represent the two-dimensional configuration emanating from the separation method: 20 Rotofor fractions were displayed from left to right (according to ascending pI) and 88 RP-HPLC fractions were displayed from top to bottom (according to ascending hydrophobicity).

High-level Expression of the PGP9.5/UCH-L1 Gene and Protein in Lung Adenocarcinoma—We further explored expression of UCH-L1 in different tumor types. UCH-L1 gene expression was analyzed in 329 tissue samples, consisting of 91 lung adenocarcinomas, 51 colon adenocarcinomas, 10 pancreatic tumors, 73 brain tumors, and 104 ovarian tumors using DNA microarrays (supplemental Fig. 2). We discovered that UCH-L1, whose protein product was found to be the target of autoantibodies in lung cancer (7), was expressed at 5- to 9-fold higher levels in lung tumors than that observed normal lung samples (p < 0.01). We further examined expression of this gene in the A549 lung adenocarcinoma cell line and found that the cell line expressed the UCH-L1 gene products at a high level.

DISCUSSION

Biomolecular approaches to the study of cancer and the discovery of genes and proteins involved in tumorigenic processes have been retarded to date because of the limitations of traditional techniques to perform large-scale analysis. Protein microarrays hold great promise to biologist and physicians as tools for performing protein expression, interaction, mapping, signaling, diagnostic, and therapeutic studies on a global scale. To date, discovery of autoantibodies has been limited by the detection technology. We have implemented a new proteomic technology as a tool for identification of cancer antigens that induce an antibody response in lung cancer. The combination of protein microarray and liquid-phase protein separation technology provides an effective means to array a wide repertoire of tumor cell proteins derived from the tumor type of interest. As a result, detection of specific interactions between tumor cell antigens in individual fractions and antibodies in patient sera is substantially facilitated. To fulfill that goal, our group elaborated a novel integral protein microarray technology.

The strategy involved, firstly, the development of an original two-dimensional IEF-RPLC system to fractionate or resolve large numbers of cellular proteins (24, 25). There is an enormous interest at the present time in developing gel-free systems for protein analysis because of their potential for multiplexing (27). Multimodular combinations of HPLC, liquid-phase IEF, and capillary electrophoresis provide various options to develop high-resolution orthogonal two-dimensional liquid phase-based strategies for the separation of complex mixtures of proteins. The separation of proteins by pl using isoelectric focusing in the first dimension and by hydrophobicity using nonporous reversed-phase HPLC in the second dimension (IEF-NP RP HPLC) is capable of resolving large numbers of cellular proteins. The improved resolution of the IEF-RPLC strategy in comparison to the classic two-dimensional gel separation, especially in the low mass and basic proteins spectra, offered us a viable alternative method for the screening of large series of protein profiles. In the present work, these protein fractions have been recovered and applied to protein microarrays to determine their antigenicity in lung cancer. Although aware of the limitations inherent to any separation technology, we consider our procedure as an integral microarray approach, in the sense that, even if not exhaustively resolved, the whole protein extract is indeed included, with a reasonably good separation of almost 2000 fractions.

The two-dimensional relationship among fractions is an important feature of our protein microarray system that can be utilized to assess reactivity of neighboring fractions in the separation process. In principle, fractions giving high intensities should cluster together with a distribution that reflects the protein separation profile. If a fraction gave high intensity but with nonreacting neighboring fractions, then such reactivity may be suspected of being an artifact. A good example for this possible false positive fraction is 0139 in Table I. However, fractions 0637 to 0641 show 4 sequential highly reactive fractions, which increases confidence that the signals are not artifacts. A built-in safeguard is the printing of fractions with a design that ensures that the adjacent fractions from the second dimension are not in the same print-tip group on a slide.

An intrinsic characteristic of remarkable consequences in our system comes from the bidimensional fractionation methodology employed. A vast majority of proteins, as we concluded for an exhaustive identification of the components of over 800 fractions by Q-TOF MS/MS analysis (results not shown), diffuse along several fractions, both in the first and second dimension. Typically, in our system, a single protein species diffuses across 3–5 fractions in the isoelectric separation and across 6–8 in the hydrophobic one, with a normal distribution profile. For a given protein, the pattern of distribution throughout fractions depends on its molecular properties, abundance levels, and stability, and it was reproducible between different experiments. This feature is of critical importance for our purposes because it determines how many and which fractions shape the response of the antibodies. Consequently, this bidimensional nature of the protein separation methodology was taken into account at the moment of the mathematical study, as most of the proteins spread across several consecutive fractions in both dimensions: instead of considering the protein of interest as confined in just
Autoantibody Detection in Lung Cancer Using Protein Arrays

In lung cancer, PGP9.5 (isoform UCH-L1) has been reported to be highly expressed (11) and to induce a humoral immune response (7), which makes that molecule susceptible to become a tumoral marker. To confirm that point, we developed a two-step strategy that involved, first, the identification of those fractions that contained the lung-specific tumor marker PGP9.5, and secondly, the specificity and sensitivity of the reaction between the antigen in those fractions and antibodies in patient sera (Fig. 1). With respect to the detection of fractions of interest, hybridization of protein microarrays with a polyclonal anti-PGP9.5 antibody revealed 15 of 1760 fractions as highly reactive. Subsequent MS/MS analyses identified the occurrence of PGP9.5 in 4 of them. Finally, a three-dimensional study on SDS gels of the selected fractions confirmed the specificity of the reaction. To our experience, several isoforms of PGP9.5 exist both in lung cancer patient samples and in lung cell lines (Fig. 3d). At least four of them can be distinctively separated by two-dimensional electrophoresis. All of them are invariably recognized by the rabbit polyclonal anti-PGP9.5 that we used in our work, and three of them are also recognized by sera from patients with lung cancer (7). However, this issue should not emerge in the protein microarray as a false positive, since all the isoforms are recognized by MS/MS as PGP9.5 variants.

Up to now, the majority of the different protein microarray techniques that have been reported involve the use of antibodies in one of the two phases of the hybridization assay, either as an immobilized probe on the surface of the slide (28, 29), or in solution (30). This comes in part because of the need of a screening device that allows for a rapid, multiplexed, low consumption assay of the levels of determined analytes in biological samples (sera, urine, saliva, etc.). The first wave of protein microarrays employed antibodies because these are the most commonly used class of protein-binding molecule available (28, 31). On the other hand, the low antibody-antigen equilibrium dissociation constants ($K_{D}$ in the $10^{-7}$-$10^{-9}$ m ranges) guarantee the highest probability of reactivity with the simplest methodology. In that sense, our experiments with anti-PGP9.5 polyclonal antibody highlighted a small group of fractions (15 fractions, <1% of total) that exhibited a significantly distinct antigenic response to it (more than 100-fold the intensity of the reactivity as compared with control sera). The finding that most of the 15 fractions were consecutive fractions, both in the first dimension (fractions no. 6, 7, 8, and 9), and in the second one (usually fractions no. 37 to 42) made us believe this might be an evidence of a “hot area” for PGP9.5 (as we explained above). For this reason, we decided to focus our following analyses in a restricted region of the microarray, where only 550 fractions from one-dimensional fractions no. 6 to 9 had been arrayed. We performed ANOVA tests on that subset of data, comparing the antibody response with normal sera response, as measured by p values and fold changes.

This strategy confirmed our previous results for most of the 15 fractions and also discovered another 5 fractions of interest. The fact that not all the 20 fractions were confirmed by MS/MS as containing the PGP9.5 antigen may be due to the higher sensitivity of the protein microarray as compared with the detection limit of the MS/MS system. The sensitivity threshold for the microarray assay is around 100 amol (1 pg of arrayed protein) recognized by a 1:100 dilution of the antibody; on the other hand, the detection limit for the configuration of the Q-TOF Micro we used is 10 fmol. In this sense, a confident identification of the molecule by MS/MS requires a higher amount of material than the detection of the antigen in the microarrays by the antibody. It is feasible, in summary, that PGP9.5 protein is distributed in several fractions, according to the chromatographic pattern of the RPLC separation, and in consequence, in some of them it is present in a lesser amount that necessary for MS/MS identification. Therefore, it will be detected by the antibody in the microarray format, but not identified by the Q-TOF. Despite this, we also assume the high incidence of false positives in these sorts of devices, as reflected in numerous previous papers (29, 32, 33).

As for the second scenario, in which we used the multifractionated arrayed cell lysates to probe lung cancer patient sera, we faced a completely different picture. Absolute intensities are not a necessary factor of consideration anymore, as the most reactive fractions will rarely contain the protein of interest (usually, a low-abundance one) but more common and highly expressed molecules, such as keratins, albumin, IgG, cytoskeleton proteins, kinases, ribosomal proteins, chaperons, etc. Because of that, we considered a selection of different mathematical criteria as a tool to discriminate those PGP9.5-reactive fractions between the almost 4000 arrayed spots. Consequently, we weighed with preference the differential reactivity for cancer sera as compared with normal individual sera. The analysis uncovered another two fractions that contained the PGP9.5 antigen that have not been previously selected against the antibody. This can be explained because of the different antibody-antigen reactivity for the serum antibody as compared with the polyclonal rabbit one. A lower amount of the antigen in the fraction or a more complex protein mixture in it may also account for a diminished response, so that the average intensity for that fraction falls below the detection threshold used for the anti-PGP9.5 antibody slides.

The protein chip results identified the occurrence of PGP9.5 autoantibodies in 5 of 14 sera from patients with lung cancer, whereas no sera from normal individuals exhibited such behavior. This analysis correlates well with previous findings, both in tumor tissues (11) and cancer sera (7), which had reported that a small group of patients with lung cancer developed a humoral immune response to PGP9.5 antigen. It is not clear why only a subset of patients with a tumor type develop a humoral response to a particular antigen. Immunogenicity may depend on the level of expression, post-trans-
lational modification, or other types of processing of a protein, the extent of which may be variable among tumors of a similar type. Variability among individuals and tumors in MCH molecules may as well be included as factors that influence the immune response.

Given the distribution profile of the chromatographic separation for a particular protein along consecutive fractions, and the expected involvement of several molecules in an even simple tumorigenic event, it could not be totally discarded that a cancer serum recognized more than one antigen in different fractions. From our comprehensive MS/MS analysis of more than 800 two-dimensional liquid fractions (results not shown), we concluded that approximately half of them contained more than one protein, and in some cases up to ten. It might occur, in theory, that more than one autoantibody response had been raised in a particular patient serum, and that the two antigens (or whatever number was) fell together in the same separative fraction. The probability of that occurrence to happen is very low, but it cannot be rejected. For that reason, we decided to validate by one-dimensional immunoblotting those positive fractions that had been confirmed by MS/MS analysis to obtain further information about the molecular weight of the reactive antigen.

PGP9.5 is widely expressed in neuronal tissues at all stages of neuronal differentiation, and that has been suggested as a neuroendocrine marker (34). In the same sense, PGP9.5 has been reported to play an essential role in sex differentiation, oogenesis, and bone marrow stromal cell differentiation. Therefore, it is not surprising that we found large PGP9.5 levels expression in brain and ovary tumors in our DNA microarray experiments. PGP9.5 has previously been associated with pulmonary neuroendocrine tumors and, less frequently, with non-small cell lung cancer (11, 35). But PGP9.5 expression in tumor tissue is not limited to lung cancer, as we can infer from the DNA expression data. For example, PGP9.5 was detected in pancreatic cancer, colorectal cancer, myeloma, and thyroid carcinoma, and it has been suggested that PGP9.5 expression may serve as a marker for predicting outcome for patients with resected pancreatic tumors, as well as in esophageal carcinoma (12, 36).

Another finding from the protein chips experiments was the apparent reactivity and specificity of the PGP9.5-containing fractions for sera from patients with colon cancer. This was at first a striking discovery because UCH-L1 (the gene that codifies for PGP9.5) is well known as being expressed neither in colon cancer nor in colon cell lines. However, as we published in a previous work (37), there exists an isotype of that protein, termed UCH-L3, that is highly expressed in colon cancer cells (as well as in lung cancer). Using a combination of colon protein microarrays and two-dimensional Western blot approaches, we demonstrated the presence of autoantibodies for UCH-L3 in 50% of sera from patients with colon cancer. Given the fact that UCH-L1 and -L3 share 64.5% of sequence identity, it is likely that cross-reactivity for both antibodies may occur. That would explain the high colon-specific reactivity of some PGP9.5 spots. According to this hypothesis, Yamazaki et al. have recently reported positive staining with polyclonal anti-PGP9.5 antibody in primary colorectal cancers, suggesting its relation to tumor progression (36).

We believe that, given the colossal predicted size and complexity of the human proteome (some estimations talk of $10^5$-$10^6$ different proteins being expressed by a given individual at a certain time), a multi-D liquid-fractionation strategy is required. Each separation step contributes to the progressive unraveling of the proteome with a $10^{-12}$ factor, offering different kinds of information about the sample according to the chosen separation ability (pH, hydrophobicity, MW, ionic strength, etc.). Liquid-handling format allows the samples to be readily accessible for further biological assays.

Each of those 1760 fractions is considered to contain between one and 10 different proteins, the estimated average amount of them being in the order of a few picograms in the arrayed spots. The approach is sensitive enough to detect specific proteins in individual fractions that have been separated through an orthogonal bidimensional system and subsequently dotted onto a nitrocellulose-supported slide. In prior microarray studies of recombinant proteins, pure protein samples spotted at concentrations of 0.1–1 mg/ml (29), or even in amounts of 10 pg per dot could be detected (38). The strategy we have developed allows detection of antigens at an estimated amount of 1 pg with an initial antigen concentration in well plates of around 1 μg/ml.

The results we have obtained suggest that microarrays that allow interrogation of thousands of proteins derived from specific tissues and cell populations can be produced. Such a tool would provide the necessary sensitivity and throughput needed for a variety of proteomics related investigations, such as identifying novel therapeutic targets and other investigations for which RNA targeted microarrays or "stripped down" recombinant protein based microarrays or antibody based microarrays are not suitable.

The technical complexity and laboriousness of this novel strategy can be seen as a drawback for a quick approximation to this methodology. However, for any laboratory proficient with proteomics techniques, and with experience enough to tackle new challenges, such as protein microarray generation, none of the steps involving our method would pose any major obstacle. Indeed, once the approach has been already implemented, the selected microarray can be constructed in less than 1 week. Taking into account the time-course planning of any study involving protein microarray devices (or even other proteomics approaches), the construction step will not be the bottleneck.

Different alternatives in the literature imply, in general, the use of a limited and much lesser number of proteins/peptides in the array, which restricts the possibilities of the device in terms of diversity, variability, and comprehensiveness. In some studies, for instance, "reverse-phase" autoantibody ar-
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rays have been described for antigen-autoantibody profiling, using a commercial platform as the starting microarray scaffold (39). In this case, the number and identity of the antigens are constrained to a few hundred; and specifically, autoantibodies anti-PGP9.5 could not have been detected because it is not one of the molecules in the array. Most of the reports using protein microarray for autoantibody profiling describe the employment of recombinant proteins (20). Nonetheless, the lack of proper post-translational modifications may generate false results when using recombinant proteins as printed antigens (22).

In brief, the authors think that our microarray platform offers some benefits for autoantibody profiling: in addition to the advantages of protein microarray technology, it brings a nearly complete and trustworthy representation of the tumoral proteome, in terms of protein ID, expression levels, post-translational modifications, distinctive characteristics of the disease, etc. This study reports the first case of protein microarrays assembled from cancer tissue derived fractions as a tool for identification of tumor antigens that induce an antibody response in different types of cancer. There is increasing evidence for an immune response to cancer in humans, demonstrated in part by the identification of autoantibodies against a number of intracellular and surface antigens in patients with different tumor types. The majority of tumor derived antigens that have been identified as eliciting a humoral response in cancer are not the products of mutated genes. They include differentiation antigens and other proteins that are overexpressed in tumors. Thus, tumor or cancer cell line-derived protein microarrays as we have developed have the capability of identifying proteins that are antigenic, as a result of their overexpression and/or post-translational modification. It is likely that such cell or tissue derived protein microarrays will have numerous other applications.

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