

2D Differential In-gel Electrophoresis for the Identification of Esophageal Scans Cell Cancer-specific Protein Markers*

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The reproducibility of conventional two-dimensional (2D) gel electrophoresis can be improved using differential in-gel electrophoresis (DIGE), a new emerging technology for proteomic analysis. In DIGE, two pools of proteins are labeled with 1-(5-carboxypentyl)-1'-propylindocarbocyanine halide (Cy3) *N*-hydroxy-succinimidyl ester and 1-(5-carboxypentyl)-1'-methylindodi-carbocyanine halide (Cy5) *N*-hydroxysuccinimidyl ester fluorescent dyes, respectively. The labeled proteins are mixed and separated in the same 2D gel. 2D DIGE was applied to quantify the differences in protein expression between laser capture microdissection-procured esophageal carcinoma cells and normal epithelial cells and to define cancer-specific and normal-specific protein markers. Analysis of the 2D images from protein lysates of ~ 250,000 cancer cells and normal cells identified 1038 protein spots in cancer cell lysates and 1088 protein spots in normal cell lysates. Of the detected proteins, 58 spots were up-regulated by >3-fold and 107 were down-regulated by >3-fold in cancer cells. In addition to previously identified down-regulated protein annexin I, tumor rejection antigen (gp96) was found up-regulated in esophageal squamous cell cancer. Global quantification of protein expression between laser capture-microdissected patient-matched cancer cells and normal cells using 2D DIGE in combination with mass spectrometry is a powerful tool for the molecular characterization of cancer progression and identification of cancer-specific protein markers. *Molecular & Cellular Proteomics* 1:117-124, 2002.

Proteomics (1) includes the systematic cataloging of protein expression on a large scale, providing complementary infor-

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mation to that obtained from mRNA profiling by microarray (2, 3). Such studies could lead to the molecular characterization of cellular events associated with cancer progression, cellular signaling, and developmental stages (4–7). Proteomics studies of clinical tumor samples have led to the identification of cancer-specific protein markers, which provide a basis for developing new methods for early diagnosis and early detection and clues to understand the molecular characterization of cancer progression (5, 8–10).

A mainstay of conventional proteomics is high resolution 2D¹ gel electrophoresis (11, 12) followed by protein identification using mass spectrometry (13–15). The state of the art 2D gel system can be loaded with a few milligrams of protein and separates thousands of protein spots (5, 16). Although the technique has been widely used and successfully applied in a variety of biological systems, several technical limitations exist. Because of subtle changes in experimental conditions, the protein expression patterns on a single 2D gel usually cannot be fully duplicated, which makes it difficult to find the proteins changed between gels and to quantify the changes in protein expression. Although a comparison of protein expression profiles from regular 2D gel electrophoresis can be carried out with the assistance of various software programs, it typically requires some computerized justification of 2D gel images so that two images can be superimposed and compared. These difficulties limit the speed and accuracy of quantitation of protein spots in 2D gel electrophoresis.

The differential in-gel electrophoresis (DIGE) technique recently introduced by Amersham Biosciences, Inc. is aimed at improving reproducibility. The concept of DIGE was originally developed by Minden and colleagues (17). To analyze the samples in DIGE, two pools of protein extracts are labeled covalently with fluorescent cyanine dyes, Cy3 and Cy5, re-

¹ The abbreviations used are: 2D, two dimensional; CHAPS, 3-[[[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Cy3, 1-(5-carboxypentyl)-1'-propylindocarbocyanine halide *N*-hydroxysuccinimidyl ester; Cy5, 1-(5-carboxypentyl)-1'-methylindodi-carbocyanine halide *N*-hydroxysuccinimidyl ester; DIGE, differential in-gel electrophoresis; HPLC, high performance liquid chromatography; MS, mass spectrometry; LCQ, liquid chromatography electrospray ion trap mass spectrometer; LCM, laser capture microdissection; DTT, dithiothreitol; 3D, three dimensional.

spectively. These labeled proteins are mixed and separated in the same 2D gel. The 2D gel patterns can be rapidly imaged by the fluorescence excitation of either Cy3 or Cy5 dyes. The amount of the dye is controlled in such a way that on average one protein molecule is labeled not more than once, and the minimum number of the molecules of each protein are labeled. A comparison of the resulting images allows quantitation of each protein spot. Because two pools of the proteins are separated in the same gel, those proteins existing in both pools will migrate to the same locations in the 2D gel, minimizing the reproducibility problem. Quantitation of the protein profile can be rapidly and accurately achieved based on the fluorescence intensity. Recently, this technique was used by Davison and colleagues (18) for proteomics studies of mouse liver homogenates to examine the molecular basis of the hepatotoxin, *N*-acetyl-*p*-aminophenol. They demonstrated that the DIGE technology has adequate sensitivity and reproducibility and wide dynamic range.

We have applied the DIGE technique for the identification of esophageal squamous cell cancer-specific protein markers. Both esophageal cancer and normal squamous epithelium cells were procured from the same esophageal tumor sample by laser capture microdissection (LCM). 200 μ g of whole cell extracts of both pools were labeled, mixed, and separated in an 18-cm \times 18-cm gel. Analysis of the produced 2D images identified 1038 protein spots in cancer cell lysate and 1088 protein spots in normal cell lysate, of which 58 protein spots were found up-regulated by >3-fold, and 107 proteins were down-regulated by >3-fold in cancer cells. Three protein spots were identified by mass spectrometry and validated by Western blotting analysis. To our knowledge, this is the first global quantitation of differential protein expression analysis by 2D-PAGE between LCM cancer cells and normal cells from the same human tumor-tissue sample.

EXPERIMENTAL PROCEDURES

Materials—Cy3, Cy5, immobilized pH gradient strips, Pharmalyte, and ECL Western blotting detection reagents were purchased from Amersham Biosciences, Inc. Urea and thiourea were bought from Fluka Chemical Corp. (Milwaukee, WI). CHAPS was obtained from Sigma. DTT was purchased from Fisher. Protease-inhibitor mixture tablets were from Roche Molecular Biochemicals. Antibodies used in this study include anti- α -tubulin and all the secondary antibodies (Sigma), anti-annexin 1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-gp96 (StressGen Biotechnology Corp., Victoria, British Columbia, Canada). The SYPRO Ruby staining kit was from Molecular Probes Inc. (Eugene, OR).

Laser Capture Microdissection—LCM (19) was performed in a Pixcell II laser capture microscope (Arcturus Engineering, Mountain View, CA). Histology of normal and malignant frozen esophageal tissue sections obtained from the same tumor sample was carefully examined by a board-certified pathologist. Frozen sections (8 μ m) were stained with hematoxylin, followed by gradual ethanol and xylene dehydration. During the period, proteinase inhibitor mixture tablets were added to the staining solutions. Once air-dried, the section was overlaid with a thermoplastic polymer film mounted on a transparent cap, and targeted cells were captured through focal melting of

the membrane by laser pulses. Laser spot size was adjusted to 30 μ m, and on average 3–5 cells were collected per laser shot. In general, 250,000 cells were procured for 2D gel electrophoresis, and 20,000–40,000 cells were collected for immunoblotting. As determined by microscopic visualization of the captured cells, the LCM enables isolation of a pure cell population (>95%).

Sample Preparation for 2D-PAGE Analysis—100 μ l of isoelectric focusing lysis solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, and protease inhibitor mixture was applied directly to the microdissected cells adhered on the LCM cap, which was then placed into an Eppendorf tube and vortexed vigorously for 5 min until all cells were completely lysed (20, 21). The isoelectric focusing lysing solution was then re-applied to another cap containing cells from the same microdissected material, and the procedure was repeated until each 100 μ l contained the whole cell lysate from \sim 250,000 cells (\sim 50,000 LCM transfer pulses). The cell lysates (200 μ g) were precipitated in 10% trichloroacetic acid/80% acetone. The precipitated proteins from LCM-procured normal and cancer cells were then dissolved in 20 mM Tris-Cl (pH 8.5), 8 M urea, 4% CHAPS, 5 mM magnesium acetate and labeled with 1.0 nmol of Cy3 and Cy5, respectively, according to the manufacturer's protocol. The labeled samples were combined and mixed with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 1% Pharmalyte (pH 3–10 NL)) before being applied to an 18-cm immobilized pH gradient strip (pH 3–10 NL) for overnight rehydration.

2D DIGE and Image Analysis—First-dimension isoelectric focusing was carried out on an Amersham Biosciences, Inc. Mulyiphor II system essentially as described by the manufacturer. Pre-cast immobilized pH gradient strips (18 cm; pH 3–10 NL) were used for the first-dimensional separation for a total focusing time of 25 kV-h. The strips were equilibrated with a solution containing 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris (pH 8.8) reduced with 100 mM DTT and directly applied to a 15% isocratic SDS-PAGE gel for electrophoresis overnight at 60-mA constant current. The Cy3-labeled gel image was collected at an excitation wavelength of 540 nm and at an emission wavelength of 590 nm whereas the Cy5-labeled gel image was collected at an excitation wavelength of 620 nm and an emission wavelength of 680 nm. The resulting gel was then visualized by SYPRO Ruby staining. The SYPRO Ruby-stained image was scanned at an excitation wavelength of 400 nm and an emission wavelength of 630 nm. All the images were collected on a 2D 2920 Master Imager (Amersham Biosciences, Inc.). Statistics and quantitation of protein expression were carried out in Decyder-DIA software (Amersham Biosciences, Inc.). Only those spots with over 3-fold changes in volume after normalization between the two populations were defined as altered.

Western Blotting Analysis—After LCM dissection, targeted cells were transferred to the lysis buffer containing 0.125 M Tris-Cl (pH 6.8), 4% SDS, 20% (v/v) glycerol, 0.2 M DTT, 0.02% bromphenol blue. The cell lysate was separated on a 12% Tris-glycine SDS-polyacrylamide gel and then transferred to nitrocellulose (Bio-Rad). Blots were blocked in 5% non-fat dry milk in Tris-buffered saline for 1 h and incubated with various primary antibodies for 1 h with constant shaking. After being washed with Tris-buffered saline (25 mM Tris-Cl (pH 7.4), 150 mM NaCl) three times, the membranes were incubated with the proper horseradish peroxidase-conjugated secondary antibodies for 45 min and developed using the enhanced chemiluminescence method (Amersham Biosciences, Inc.). To evaluate the expression level of different proteins in paired cases, protein loads were normalized by Western blotting with the antibody against α -tubulin and/or 0.2% Ponceau staining.

Mass Spectrometry Analysis—The separated proteins in SDS-PAGE gels were visualized by SYPRO Ruby staining. The spots of interest were excised, in-gel digested, and extracted as described previously (22). The dried peptide sample was dissolved in 6 μ l of HPLC Buffer A solution (water:acetonitrile:acetic acid, 97.5:2:0.5

(v/v/v)) for mass analysis. HPLC-MS/MS analysis was performed in an LCQ (ThermoFinnigan, San Jose, CA) coupled on-line with an HPLC system (SMART System; Amersham Biosciences, Inc.). 2 μ l of protein digests obtained above were loaded on the HPLC connected with an in-house packed C18 column (~5–10-cm-length; 75- μ m inner diameter). To reduce the dead volume, a high voltage (1.2 to 1.4 kV) was applied to the HPLC side of the column through a zero dead volume three-way Y connector (Valco Instruments Co., Inc., Houston, TX).

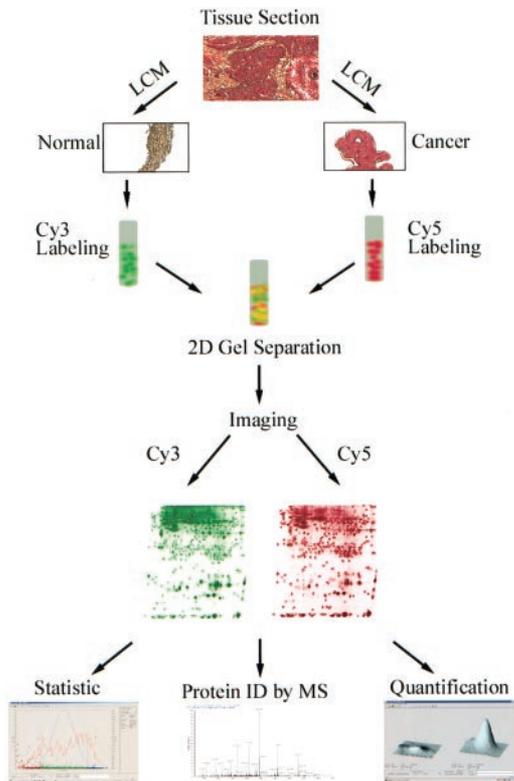


FIG. 1. Flow chart of DIGE analysis of procured normal cells and cancer cells from the same tumor sample. ID, identification.

The peptides were sequentially eluted from the HPLC column with a gradient of 5 to 90% of Buffer B (acetonitrile:water:acetic acid, 90:9.5:0.5) in Buffer A (acetonitrile:water:acetic acid, 2:97.5:0.5) at a flow rate of ~0.7 μ l/min. The eluted peptides were sprayed directly from the tip of the capillary column to the LCQ mass spectrometer for mass spectrometry analysis. The LCQ was operated in a data-dependent mode where the machine measured intensity of all peptide ions in the mass range of 400 to 1400 (mass-to-charge ratios) and isolated the peptide peak with the highest intensity for collision-induced dissociation. In this way, masses of both the parent peptide and its daughter ions were detected. The accurately measured masses of the tryptic peptide and its fragments were used to search for protein candidates in the protein sequence data base with the program Knexus (Proteometrics LLC., New York, NY).

The experimental procedures are illustrated as a flow chart and are presented in Fig. 1.

RESULTS

2D Gel Separation of Proteins—~250,000 normal and cancer cells were procured from the same esophageal carcinoma sample. Whole cell lysates (200 μ g) were produced and precipitated by trichloroacetic acid/acetone. The precipitation eliminated lipids, nucleotides, and salts, which improves the resolution of 2D gel analysis (12, 23, 24). The precipitated proteins from both normal cells and cancer cells were labeled with Cy3 and Cy5 dyes, respectively, according to the manufacturer's protocol. The labeling conditions ensured that less than 5% of molecules of each protein were labeled. The resulting two pools of proteins were mixed and subjected to isoelectric focusing in a Multiphor II apparatus, followed by 15% SDS-PAGE in the 2nd dimension.

2D gel images of cancer cells (Cy5 image; see Fig. 2A) and normal cells (Cy3 image; see Fig. 2B) were produced in a 2D 2920 Master Imager. The generation of the images can be completed in ~20 min, depending on the exposure time.

The Changes of Protein Expression Patterns between Cy3- and Cy5-imaged Gels—Comparisons of protein expression in

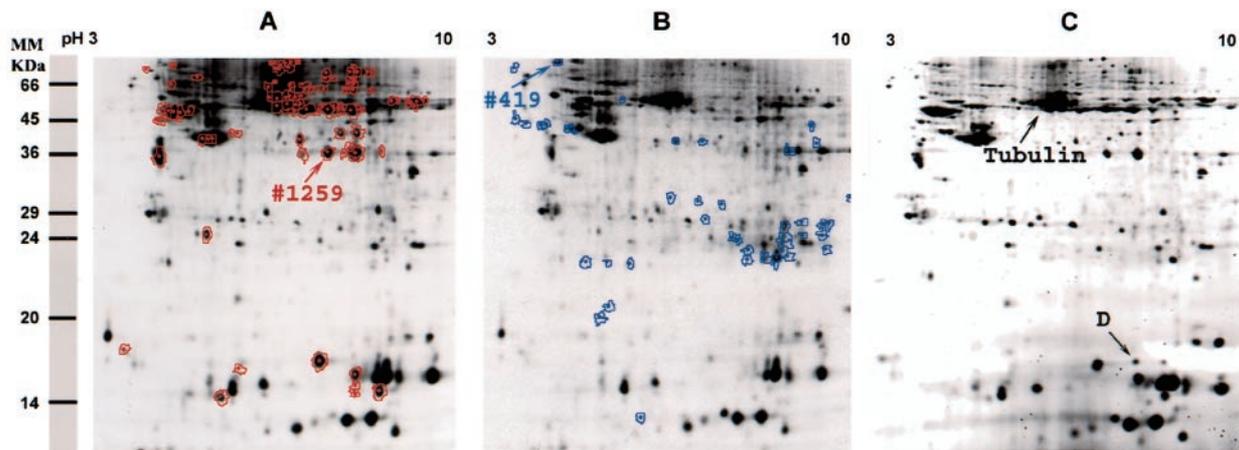


FIG. 2. DIGE gel images of normal and cancer cells. A, Cy3 image of proteins from normal cells; B, Cy5 image of proteins from tumor cells; C, SYPRO Ruby-stained gel image. The labeled normal and cancer cell proteins were mixed and separated in an 18 \times 18-cm gel. The same gel stained by SYPRO Ruby (C) gave the combined protein profile of both normal cells and cancer cells. The subtle difference of protein profiles between A and C or B and C resulted from the changes of molecular masses (MM) (~0.5 kDa) after labeling reactions and differential labeling efficiency among proteins because of the difference in lysine content such as spot D.

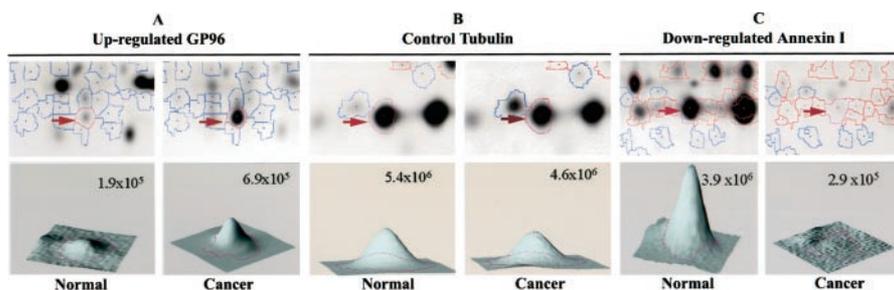
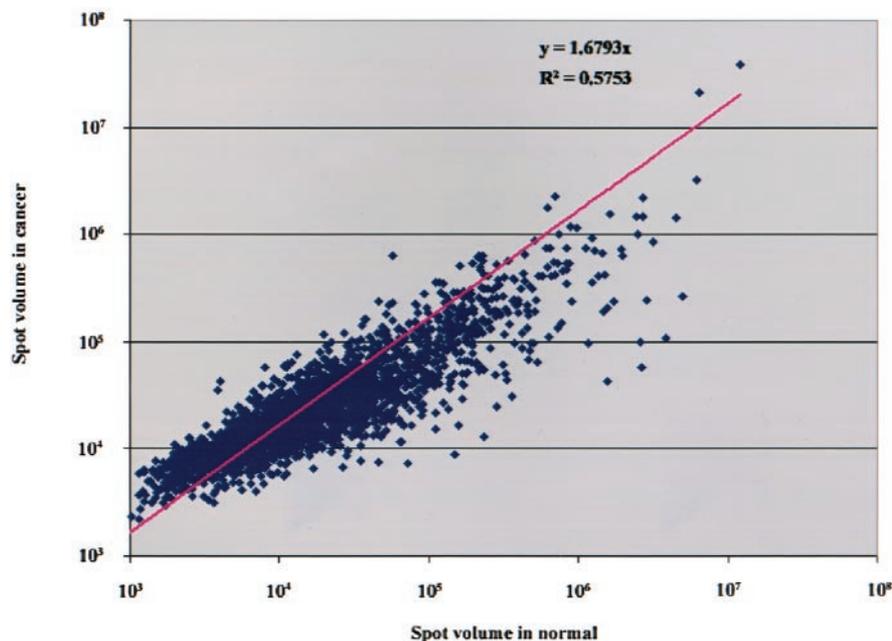


FIG. 3. **3D simulation of up- and down-regulated protein spots.** The volume of each spot was calculated using Decyder-DIA software and is graphically represented. The spot pairs of up-regulated (A), equally expressed (B), and down-regulated (C) proteins in cancer cells were shown. The *upper panel* shows the images of the protein spots in the 2D gel, and the *lower panel* shows the 3D image of the corresponding spots and their calculated spot volumes. The amount of the protein is proportional to the volume of the protein peak.

FIG. 4. **Scattergram depiction of DIGE data to generate protein expression profile.** Each *data point* represents spot volumes of the Cy3 and Cy5 images. The *x axis* indicates the volumes of protein spots in normal cells whereas the *y axis* represents those in cancer cells. The protein expression profile showed no significant difference between cancer cells and normal cells and correlated to a linear distribution ($y = 1.68x$; $R^2 = 0.57$).



2D images were carried out using Decyder-DIA software. An analysis of Cy5- and Cy3-imaged gels identified 1038 protein spots in cancer cells and 1088 protein spots in normal cells when 20,000 pixels were used as the filter limit. The pixel volume of each spot was calculated based on spot intensity and spot area and was followed by the normalization with the total pixel volume of all the spots in the gel image. The pixel volume of each spot provides the basis for comparison of protein expression between cancer cells and normal cells (Fig. 3). As shown in the scatter plot (Fig. 4), the volume of paired protein spots showed a linear distribution. The correlation coefficient between cancer and normal was 0.75.

The *t* test was used to compare the overall protein expression profile. There was no overall significant difference between cancer and normal images ($n = 1264$; $p > 0.05$). Of the 1264 spots detected in two images, 492 spots were shown decreased, and 745 increased in cancer. The mean of decreased population was -1.26 with the S.D. = 2.83 whereas the mean of the increased population was 0.85 with the S.D. =

0.83. 39 protein spots were shown to be statistically significantly decreased (over 4-fold; $p < 0.05$; single tail), and 56 protein spots increased (>3.5 -fold; $p < 0.05$; single tail) in cancer cells.

Quantitation of Protein Expression in Decyder-DIA Software—Analysis of the protein expression using Decyder-DIA software provided 3D simulation of the protein spots, allowing an objective view for the comparison of spot intensity between the two images. Each protein spot could be presented in 3D views by its relative amount and distribution. The 3D peak of a protein spot was generated based on the pixel *versus* area data from the images obtained by the 2D Master Imager. The peak area showed the distribution of the protein spot in the gel whereas the volume correlated to the protein amount. Each pair of protein spots (Cy3- and Cy5-labeled) could be presented in 3D views by their relative peak volumes, which were calculated from the volume of the spot normalized by the total volume of the image. The comparison of the spot intensities is relatively more objective this way than the conventional way where brightness/contrast is adjusted manually

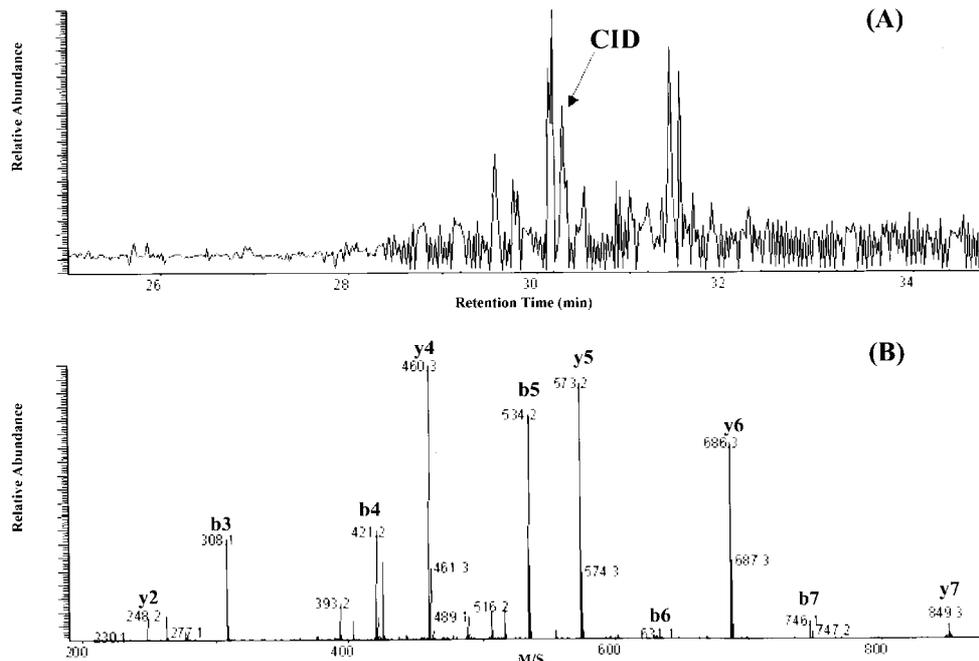


FIG. 5. Protein identification by mass spectrometry. A, HPLC profile of HPLC/MS/MS analysis. The x axis represents the retention time of the analysis, and the y axis represents ion current detected. The machine is set such that both masses of parent peptide and fragments of the strongest tryptic peptide are determined. *CID*, collision-induced dissociation. B, MS/MS analysis of the peptide ions with a mass-to-charge ratio of 498 at the retention time of 30.3 min. The peptide was isolated and fragmented in the LCQ, leading to mass information of the fragments. A search of the NCBI non-redundancy protein sequence data base using the obtained masses of peptide fragments identified a peptide sequence SGYLLPDTK, which is unique to tumor rejection antigen-1. *b* and *y* designate the N- and C-terminal fragments of the peptide produced by breakage at the peptide bond in LCQ, respectively. The *number* represents the residue number from either the N or C terminus.

to the same degree. As shown in Fig. 3A, spot 419, identified as tumor rejection antigen gp96, was up-regulated over 3-fold in tumor tissue, and in Fig. 3C, spot 259, identified as annexin I, was down-regulated in tumor tissue. Because each peak was scaled to fit the window for the 3D image, the actual spot intensity was represented by peak volume rather than peak height as shown in the figure.

Among the protein spots detected, 58 protein spots were up-regulated by >3-fold, and 107 were down-regulated by >3-fold. No significant changes (less than 2-fold) were observed among 916 protein spots (~72.5% of the detected proteins). All the quantitation and statistics can be completed in 30 min, suggesting the advantage of the DIGE technique.

Comparison of Protein Expression Patterns between Cy3 and Cy5 Images and SYPRO Ruby-stained Gel Images—To evaluate the global changes of protein pattern caused by dye labeling, the same gel was also stained with SYPRO Ruby. The Cy3 and Cy5 images, originally from the same gel, could be superimposed and compared directly without any warping. The overall protein-staining patterns between the SYPRO Ruby image and the dye images were very similar. However, there are subtle differences between the SYPRO Ruby image and the dye-labeled images, which could be caused by two major factors: (1) the molecular mass of the labeled proteins was typically shifted up in Cy3 or Cy5 images, because the dyes added ~0.5 kDa to the total molecular mass, and (2) the

abundance of protein spots in Cy3 or Cy5 images could be either increased or decreased depending on the high/low abundance of lysine composition in the proteins. For example, a clear spot D in the SYPRO Ruby image (Fig. 2C) disappeared in either the Cy5 or Cy3 images (Fig. 2, A and B).

Protein Identification by Capillary HPLC/MS/MS—Because the labeled protein has a molecular mass ~0.5 kDa higher than the unmodified protein, and the minimum number of molecules of each protein were labeled, the unlabeled protein spot rather than the labeled protein spot was excised for mass spectrometry analysis. The protein of interest (up- or down-regulated) was defined from the Cy3 or Cy5 images, and its corresponding spot in the SYPRO Ruby image was matched. The protein spot was excised manually and subjected to in-gel digestion and capillary HPLC tandem mass analysis in an LCQ mass spectrometer. The machine determined the molecular masses of tryptic peptides eluted from the HPLC column and their fragments, which are used to identify the protein by matching to known protein sequences existing in the NCBI non-redundancy protein sequence data base and EST sequence data base. The protein identification was assisted by Knexus software, and each search was completed in <3 min. We are able to identify any protein spots that could be visualized by SYPRO Ruby staining.

Fig. 5A shows the HPLC/MS/MS analysis of tryptic peptides of spot 419 (Fig. 2B). Fragmentation of the peptide with

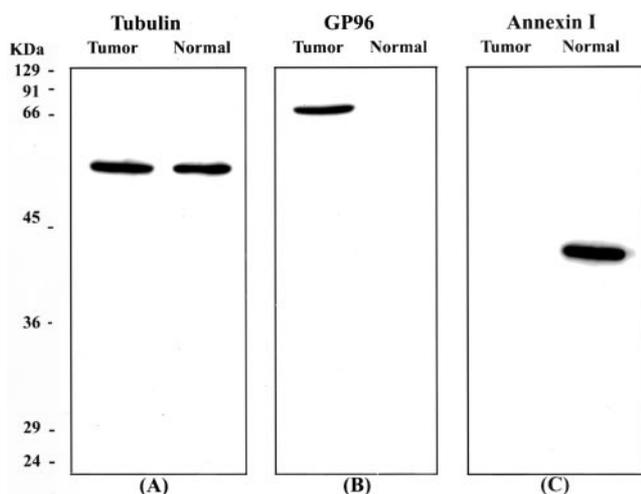


FIG. 6. Validation of the identified proteins by Western blotting analysis. 40,000 cells were microdissected, lysed, and separated in a 12% SDS-PAGE gel, which was probed by the antibodies against α -tubulin (A), gp96 (B), and annexin I (C). A, normalization of protein loading by antibody against α -tubulin (1:1000 dilution). B, loss of annexin I (1:1000 dilution). C, increased expression level of gp96 (1:2000 dilution) in squamous cell carcinoma of esophagus.

a doubly charged mass of 498 determined the masses of the peptide daughter ions (Fig. 5B). A data base search using the mass of the parent peptide and its fragment ions with Knexus software identified peptide sequence SGYLLPDTK, unique to tumor rejection antigen (gp96). Annexin I (spot 1259; see Fig. 2A) and α -tubulin were identified likewise.

Confirmation of the Identified Proteins by Western Blotting Analysis—To ensure the identified proteins were truly overexpressed or underexpressed in cancer, a small portion of the protein lysates were subjected to one-dimensional SDS-PAGE and blotted with the antibodies against the control protein α -tubulin, up-regulated protein tumor rejection antigen (gp96), and down-regulated protein annexin I (Fig. 6). As expected, no change was observed for tubulin between cancer cells and normal cells. However, for the antibody against protein gp96, a clear band, corresponding to the molecular mass of the protein gp96, was observed in tumor lysates but not in normal cell lysates, suggesting the absence of the protein in normal cells. In contrary, protein annexin I was only observed in normal cells but not in tumor cells. Thus, Western blotting experiments confirmed the results from 2D gel analysis and protein identification by mass spectrometry.

DISCUSSION

DIGE is a new approach in comparative differential display proteomics. Previously this technology has not been applied to direct analysis of diseased cells in human tissue. We have successfully applied the DIGE technique to the identification of cancer-specific protein markers from LCM of human esophageal tissue. This analysis identified 58 protein spots that were overexpressed and 107 proteins that were down-regulated greater than 3-fold in cancer cells. Selected pro-

teins with greater than 3-fold change in cancer versus normal were identified by capillary HPLC mass spectrometry and further confirmed by Western blotting analysis to validate the differential expression identified by DIGE.

Proteomic analysis of a human cancer employing the 2D DIGE technique highlights several advantages. First, because the two pools of protein extracts were separated in the same gel, the reproducibility compared with conventional 2D gel separation is improved, and the comparison of protein expression patterns is simplified. Second, the differences in protein expression between two populations of proteins can be more accurately imaged, and differential protein expression can be easily identified, based on fluorescence of the labeled Cy3 and Cy5 dyes, thus providing accurate quantitation of protein changes. Because fluorescence enables quantitation of protein spots/bands with a dynamic range of four orders of magnitude (25), the technique has wider dynamic range than more conventional staining methods such as silver staining, Coomassie Blue, and colloidal Coomassie Blue. Third, DIGE requires less time to detect the protein spots. The labeling reaction in DIGE takes about 45 min, which is faster than other staining methods with similar detection sensitivity, such as silver staining (>1 h) and SYPRO Ruby staining (>3 h). Finally, DIGE represents a step forward for high throughput analysis of 2D gels by its capability for automatic gel imaging and easy quantitation and comparison of gel images and because 50% fewer gels are required for analysis (because two pools of samples are separated in the same gel). The technique allows protein quantitation of two pools of protein profiles in less than 30 min using our 1-gigabyte processor.

The 2D DIGE method exhibited high reproducibility. In an independent experiment (data not shown), aliquots of 75 μ g of B cell lysates were labeled with Cy3 or Cy5, respectively. 2D DIGE analysis revealed four of 1100 spots with statistically significant differences between the gels. Two spots were increased in the Cy3 image, and the other two were increased in the Cy5 image. There were no significant changes among six parallel gels loaded with the same samples ($p > 0.05$ by Student's *t* test).

Labeling of proteins by fluorescent dyes did not affect the protein identification by mass spectrometry. Because only small percentages of the molecules of each protein are labeled, we typically did not observe the dye-labeled proteolytic peptides in mass spectrometry because of the limited labeling and limited dynamic range of mass spectrometric analysis.

Although the technique is very useful, a few technical details need to be considered. First, the protein patterns obtained with the DIGE system could be different from those obtained with conventional systems. The technique relies on the fluorescence dye for quantitation of proteins; therefore, critical control of labeling reaction conditions will be essential for accurate and quantitative results. Proteins will be labeled with different efficiencies depending on lysine content. The same protein in two pools of protein extracts could be labeled

with similar efficiency as long as the reaction conditions are well controlled. However, those proteins with a high percentage of lysine residues could be labeled more efficiently compared with proteins containing little or no lysine. In addition, the technique is not applicable to those proteins without lysine. Therefore, a high abundance protein spot in a conventional gel system could be a medium or low abundance protein spot in the DIGE system because of its low lysine content.

Moreover, the labeling reaction slightly changes the location of protein spots in a 2D image. Although the covalently linked dye will not change the pI values of the proteins, because the dye molecule has a positive charge to compensate the charge on the lysine group, it adds ~0.5 kDa to the total molecular mass of the protein. Therefore, the labeled protein could migrate in higher position in the 2nd dimension of the 2D gel than its corresponding unlabeled protein. This migration will be more significant for small proteins than for large proteins. Because only a small percentage of the protein is labeled, most of the protein molecules cannot be visualized in the Cy3 or Cy5 images. To locate the unlabeled protein, the 2D gel is visualized by SYPRO Ruby staining, and the protein of interest is identified by comparison of the patterns of the SYPRO Ruby-stained image and the Cy3 and Cy5 images.

In summary, we report the feasible use of an emerging proteomic technology, 2D DIGE, as demonstrated by the application of this tool for profiling and identification of cancer-specific protein markers using laser capture-microdissected human tissue cells. This technique appears to have advantages of adequate sensitivity, high reproducibility, and a wide dynamic range. This technique will be very useful for those applications that require accurate quantitation and direct differential proteomic analysis of normal and diseased cell populations and permits large scale analysis of clinical tissue samples.

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