

An Approach to Proteomic Analysis of Human Tumors[†]

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A strategy for proteomic analysis of microdissected cells derived from human tumor specimens is described and demonstrated by using esophageal cancer as an example. Normal squamous epithelium and corresponding tumor cells from two patients were procured by laser-capture microdissection and studied by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Fifty thousand cells resolved approximately 675 distinct proteins (or isoforms) with molecular weights ranging between 10 and 200 kDa and isoelectric points of pH 3–10. Comparison of the microdissected protein profiles showed a high degree of similarity between the matched normal-tumor samples (98% identical). However, 17 proteins showed tumor-specific alterations, including 10 that were uniquely present in the tumors and seven that were observed only in the normal epithelium. Two of the altered proteins were characterized by mass spectrometry and immunoblot analysis and were identified as cytokeratin 1 and annexin I. Acquisition of 2D-PAGE protein profiles, visualization of dysregulated proteins, and subsequent determination of the identity of selected proteins through high-sensitivity MS-MS microsequencing are possible from microdissected cell populations. These separation and analytical techniques are uniquely capable of detecting tumor-specific alterations. Continued refinement of techniques and methodologies to determine the abundance and status of proteins in vivo holds great promise for future study of normal cells and associated neoplasms. *Mol. Carcinog.* 27:158–165, 2000. Published by Wiley-Liss Inc.

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INTRODUCTION

Proteomic studies provide an understanding of cellular behavior in terms of abundance and status of proteins [1]. This approach can be applied to multiple biological systems from development of simple model organisms to disease progression in humans. As an example, precise and accurate knowledge of the repertoire of proteins associated with human cancers will likely provide insights into the fundamental mechanisms of tumor progression, and/or provide new drug targets, vaccine antigens, or markers for early detection. However, there are several technical challenges and caveats, which must be considered for studies of human tissue specimens. Previous proteomics efforts have primarily utilized tumor cell lines or bulk tissue samples as template for analysis [2–10]. While these efforts have been successful and have produced findings of interest, the results need to be inter-

preted with caution owing to the unknown effects of tissue culture on protein profiles and the mixed population of cells which inevitably exist in bulk tissue samples. Moreover, in general, these studies have not included direct intrapatient comparison of the tumor cells and the normal epithelial cells from which they arose, thus the contribution of interpatient variability to protein profiles is not clear.

In the present study, we have assessed the feasibility of proteomic analysis of histopathologically-defined cell populations from tumor samples.

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Abbreviations: LCM, laser-capture microdissection; 2-D PAGE; two-dimensional polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; 2D, two-dimensional; 1D-PAGE, one-dimensional polyacrylamide gel electrophoresis.

Normal squamous esophageal epithelial cells, stromal cells, and tumor cells from two patients were subjected to laser-capture microdissection (LCM), and the recovered proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Protein fingerprints of each population were compared, and several esophageal tumor-specific and normal epithelium-specific protein alterations were identified. More significantly, the study demonstrates a successful strategy and provides methodologies for proteomic analysis of microdissected normal and matching tumor cell populations from tissue samples, for rapid and efficient microdissection, and for determination of the identity of dysregulated proteins.

METHODS

Patients and Tissue Samples

The two specimens studied were from patients who had visited the Shanxi cancer Hospital in Taiyuan, Shanxi Province, People's Republic of China and were diagnosed with esophageal cancer. Both patients were considered candidates for curative surgical resection. The study was approved by the Institutional Review Board of the Shanxi Cancer Hospital and the U.S. National Cancer Institute. Both cases were stage II squamous cell carcinomas of the esophagus.

Microdissection

Frozen section slides were prepared from each case and microdissected by LCM (Pixcell 100, Arcturus Engineering, Mountain View, CA) as previously described except that AEBSEF (Boehringer Mannheim) was added to the staining baths at a final concentration of 2 mM to inhibit proteases [11,12]. In each case, 50 000 cells were procured. Based on a careful review of the histologic sections, each microdissection was estimated to contain >98% of desired cells.

Sample Preparation

One hundred microliters of IEF lysing solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% MEGA-10, 1% octyl- β -glucopyranoside, 40 mM Tris, 50 mM dithiothreitol, and 2 mM tributylphosphine and 0.5% (v/v) Pharmalytes was applied directly to the microdissected cells adhered on the LCM cap; placed into an Eppendorf tube; and vortexed vigorously for 1 min until all cells were completely lysed. The IEF lysing solution was then reapplied to another cap holding cells from the same microdissected case, and the procedure was repeated until each 100 μ L contained lysate from 50 000 cells (approximately 7000 LCM transfer pulses).

2D-PAGE and Image Analysis

First-dimension isoelectric focusing was carried out on a Pharmacia Immobiline IPG Dry-strip

system essentially as described by the manufacturer. Precast immobilized pH gradient strips (18 cm, 3–10 nonlinear) were employed for the first dimensional separation for a total focusing time of 120 kV-h. The strips were reequilibrated with a solution containing sodium dodecyl sulfate (SDS) and Tris (pH 6.9), reduced with tributylphosphine (2 mM), alkylated with iodoacetamide (2.5% w/v), and directly applied to a 9–18% linear gradient SDS-polyacrylamide gel for electrophoresis overnight at 40 V constant voltage. The gels were stained with silver, and direct scanning and image analysis were performed by using an Umax scanner with Adobe Photoshop software and Tektronix IISDX photographic-quality printer. Scanned images (saved as TIF files) were analyzed and compared by using the MELANIE II software package (BioRad). Protein spots (features) were detected by using the default algorithm (Laplacian plus feature shapes), and four landmark proteins were chosen that were represented reproducibly on all gels chosen for further analysis. Each gel was independently chosen as a reference gel and compared against the paired normal or tumor two-dimensional (2D) image. Alignment and matching for each was then performed by using the default pairing parameters in the software package. Comparisons of protein fingerprints were performed by using images representing protein spots readily apparent by direct visualization. Only those spots that were present or completely absent between normal-tumor cells were defined as altered. Each experiment was performed in duplicate and produced similar results (data not shown). Normalization of sample load was by anti- α -tubulin immunoblot analysis prior to the first-dimension run (Figure 1C). Scoring of the blots included comparison of multiple exposure times.

Analysis of α -Tubulin

Ten microliters of the IEF lysate was diluted 1:1 in 2 \times SDS sample buffer, boiled for 5 min, and applied to a 4–20% NOVEX Tris-glycine SDS gel and electrophoresed for 1 h. Immunoblotting was performed for 2.5 h by using a Bio-Rad semi-dry blotting apparatus with an Immobilon PVDF membrane as the capture surface. Blots were blocked with 1 \times Tris-buffered saline containing 1% ovalbumin and incubated with a monoclonal anti- α -tubulin antibody for 3 h. Anti- α -tubulin antibodies were purchased from Sigma and used at final dilution of 1:1000. Blots were washed with 1 \times Tris-buffered saline three times for 5 min and secondary antibody was added. Horse raddish peroxidase-coupled rabbit anti-mouse secondary antibodies were purchased from Sigma and used at a final dilution of 1:10 000. Blots were washed and ECL substrate (Amersham) was added for chemiluminescent detection via autoradiography on Kodak Bio-Max film.

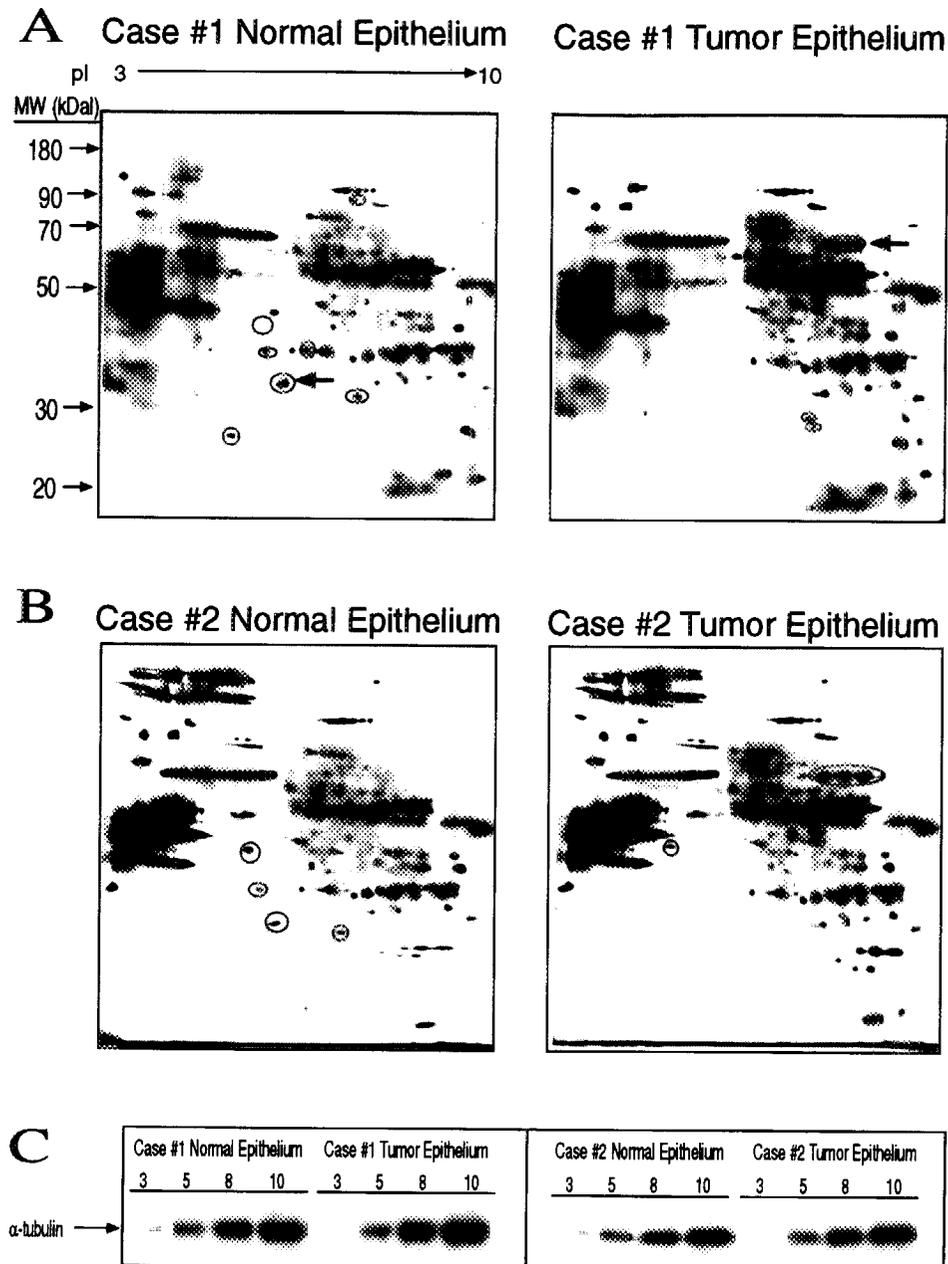


Figure 1. 2D-PAGE comparison of microdissected normal squamous epithelium and tumor cells. Fifty thousand cells were procured by LCM, directly lysed in IEF buffer, and run on a 3–10 NL Pharmacia IPG IEF strip for 100 kV. The second dimension runs were performed on 8–18% linear gradient SDS–PAGE gels, and the gels were stained with silver. (A and B) Matched tumor and normal fingerprints for each patient. Normal and/or tumor-specific proteins found repro-

ducibly are circled. The two proteins delineated with the arrowheads were chosen for further protein characterization. A representative pI and molecular weight ruler for direct comparison and alignment is shown in panel A. (C) An α -tubulin immunoblot was used to normalize for relative protein loads with 10, 8, 5, and 3 μ L of the sample loaded.

Mass Spectrometry

A separate 2D-PAGE gel was run with microdissected tumor cells from case #1 except that the gel was stained and analyzed with zinc imidazole [13]. Two proteins that were aberrantly regulated in both tumors were chosen for mass spectrometry MS-MS sequencing. The acrylamide plug was taken from the gel and washed once with high-pressure-liquid

chromatography-grade water for 5 min and subjected to in-gel tryptic digestion as previously described [13].

In-Gel Proteolytic Digestion

Gel pieces were excised and washed by end-over-end mixing in 12 mL of 30% methanol for 30 min (room temperature) and washed twice for 30 min with 150 μ L of 1:1 acetonitrile/100 mM ammonium

bicarbonate, pH 8. Each spot was sliced into one-fourths and rehydrated in 10 μ L of 100 mM ammonium bicarbonate, pH 8, containing trypsin (2 pmol/ μ L) (Promega modified trypsin). An additional 10 μ L of digestion buffer without trypsin was then added. After incubation at 37°C for 20 h, the condensate was collected by spinning the tubes briefly and removing the excess liquid into a new tube. Peptides remaining in the gel matrix were extracted twice with 150 μ L of 60% aqueous acetonitrile and 0.1% TFA at 30°C for 30 min. The extracted volume was reduced to about 5 μ L by using an Eppendorf speed-vac concentrator [14].

Electrospray Mass Spectrometry

Mass spectrometric data regarding tryptic peptides from in-gel digestions were obtained from collision-induced dissociation spectra with a Finnigan-MAT LCQ ion trap mass spectrometer after introduction via a polyamide coated fused silica microcapillary [15–17].

Immunoblot Analysis of Cytokeratin 1 and Annexin I

Two 2D gels were run simultaneously, both containing identical amounts of lysates of microdissected tumor from case #1. One gel was silver stained and the other was immunoblotted to a PVDF membrane as outlined above. 2D western blot analysis was performed as described earlier except that antibody to type II cytokeratin or annexin I was used as a probe. Anti-pan type II cytokeratin antibody was purchased from Sigma and used at a final dilution of 1:1000. Anti-annexin I antibody was purchased from Transduction Labs and used at a final dilution of 1:5000.

RESULTS

Protein Profiles of Matched Normal and Tumor Esophageal Cells

Protein profiles from microdissected normal epithelium and tumor cells from two patients were analyzed by 2D-PAGE (Figure 1). For the purpose of this study proteins were scored as “altered” only if there was a clear-cut “on-off” difference between the comparison groups. We did not attempt to quantitatively evaluate the level of those proteins

that were present in both sample groups and showed subtle differences in levels. Immunoblot analysis of α -tubulin by using a small aliquot of each sample was used to verify that equal amounts of total protein were analyzed from each dissection (Figure 1C).

The overall protein profiles between the normal-tumor pairs were highly similar. For example, the microdissected normal epithelium from case #1 shared 98% of the observed proteins with the corresponding tumor (Table 1). However, 17 distinct differences were observed between the paired normal-tumor samples, 12 of which occurred identically in both cases. The circled spots in Figure 1A and B show proteins that are either resolated or down-regulated in each tumor as compared with the matching normal epithelium. As a control to assess inherent 2D-PAGE variability, each experiment was performed twice and the results compared. One hundred percent of proteins were scored as identical in each duplicate run (results not shown).

Whole-Tissue Sections versus Microdissected Epithelial Cells versus Stroma

Samples from whole tissue sections prepared without dissection were analyzed by 2D-PAGE and compared with microdissected normal epithelial and tumor cell populations from the same section. The experiment was performed for two purposes: to assess the impact of potential LCM-induced modifications such as protein oxidation and proteolysis on 2D-PAGE fingerprints and to determine the percentage of proteins present in a microdissected cell population that could be reliably identified in the whole-tissue section from which they were derived. Figure 2 shows the 2D-PAGE comparison of the undissected whole tissue section and corresponding microdissected samples. All of the proteins in the 2D gel from the microdissected cells, including those found to be differentially regulated in the tumor or normal populations (circled), were visualized at the same Mr and pl in the undissected whole-tissue section, indicating that LCM had no apparent effect on proteins recovered from microdissected cells and reinforcing the observation that the process of LCM does not seem to effect the protein migration on 2D-PAGE [18].

Table 1. Comparison of Protein Profiles of Microdissected Normal Epithelial Cells and Tumor Cells from Two Esophageal Cancer Cases*

	# Protein alterations
Case #1 Normal epithelium vs. Case #1 Tumor	15 (2%)
Case #2 Normal epithelium vs. Case #2 Tumor	14 (2%)
Case #1 Normal epithelium vs. Case #1 Tumor	525 (78%)

*Analysis of stromal cells was also performed for case #1. Protein differences are listed as the absolute number of altered proteins between the comparison groups. The number of alterations as the percentage of total observed proteins is listed in parentheses.

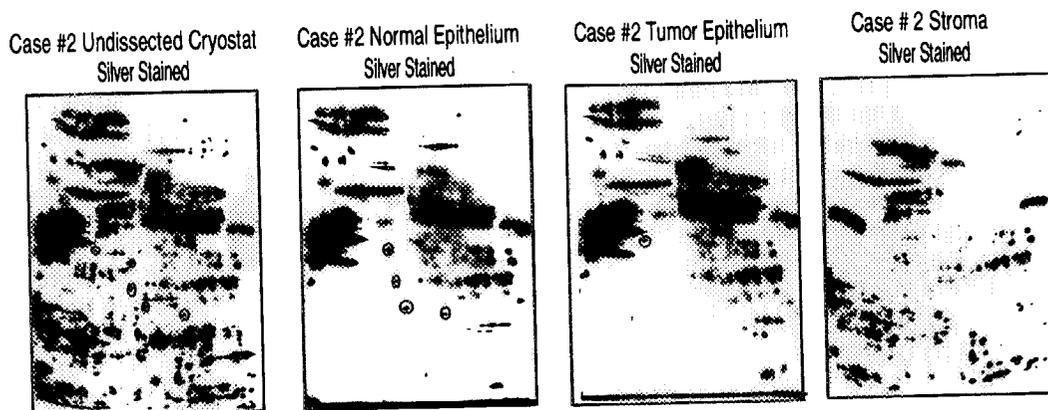


Figure 2. 2D-PAGE comparison of undisseminated tissue section (whole cryostat) and LCM-dissected epithelial and stromal cells. Lysate derived from an undisseminated whole tissue section was normalized to LCM-acquired material from the same case by using α -tubulin immunoblot analysis as an internal control. Fifty thousand cells of normal and tumor epithelial cells and stromal cells from the

same tissue section were procured by LCM, directly lysed in IEF buffer, and run on a 3–10 NL Pharmacia IPG IEF strip for 100 kV-h. The second-dimension runs were performed on 9–18% linear gradient SDS-polyacrylamide gels, and the gels were stained with silver.

To ensure that the present approach is efficient in detecting protein alterations between microdissected cell populations, a region of stroma was microdissected from the same patient and compared with patient-matched dissected normal epithelium and tumor (Figure 2). Stroma cells represent a lineage and phenotype markedly distinct from epithelium. Less than 25% (approximately 150 of 675) of the observed proteins were scored as identical between the two populations (Table 1). None of the 17 proteins that were altered in the normal epithelium–tumor comparisons were present in the stroma, indicating that these proteins were epithelial specific and highlight the impact of microdissection on final differential analysis. Furthermore, a comparison between the 2D-PAGE protein profile obtained from undisseminated cryostat tissue section and that of the protein pattern seen in the epithelial and stromal dissected samples revealed that the proteins found specifically expressed in each of the specific lineages were seen in the undisseminated protein fingerprint.

Protein Identification

Two proteins, one overexpressed and the other underexpressed in both tumors, were selected for further analysis to determine the feasibility of obtaining identification of proteins derived from microdissected cells. The spots on the 2D gel marked with an arrow in Figure 1 were chosen for mass spectrometry MS-MS sequencing. A second 2D-PAGE gel was run except that the gel was stained and analyzed with zinc-imidazole followed by protein elution and in-gel digestion with trypsin [13]. By matching the experimental molecular weights of trypsin digestion products defined by MS-MS analysis with theoretical predictions as shown in Table 2, we were able to identify multiple peptides for each of the two proteins, spanning

Table 2. Protein Identification by Mass Spectrometry*

Residues	Mass found	Mass calculated
Cytokeratin I		
278–288	1265.0	1265.63
444–455	1358.2	1357.7
212–223	1476.06	1475.75
418–432	1717.94	1716.85
Annexin I		
128–143	1702.88	1703.6
98–112	1605.96	1607.54
214–227	1550.82	1551.46
58–70	1387.76	1388.00

*The two protein spots indicated by an asterisk in Figure 1 were excised and pooled for in-gel digestion. For each sample, the peptide mass peaks in the ESI-MS spectra were easily distinguished. A large number of authentic peptide mass peaks were present, and this made identification by peptide-matching computer programs straightforward and reliable.

broad regions of the primary amino acid sequence [16,17]. The protein overexpressed in tumor was identified as cytokeratin 1, and the protein underexpressed in tumor was identified as annexin I. The identities of both proteins were confirmed by immunoblots of lysates from microdissected cells electrophoresed by either 2D-PAGE or one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) (Figure 3).

DISCUSSION

Previous studies of microdissected cell populations from tumor samples have focused primarily on DNA, and more recently on analyses of mRNA, and have been aimed at identifying gross genomic alterations, specific genes with mutations, or mRNA levels [19–26]. While these efforts have been successful and hold great promise for identifying

^{125}I labeling or biotinylation dramatically increase the number of proteins visualized from microdissected cells (data not shown). Similarly, scanning immunoblotting with class-specific antibodies allows for sensitive detection of specific subsets of proteins, for example, all known proteins involved in cell-cycle regulation [30].

However, highly sensitive protein fingerprints are problematic because of the small amount of material analyzed and subsequent difficulty in identifying proteins of interest. Therefore, a useful strategy is to produce parallel "diagnostic" and "sequencing" 2D gels from each case. The diagnostic fingerprints are derived from microdissected cells and provide maximal sensitivity for detection of normal-tumor differences. Sequencing fingerprints then allow for determination of protein identity. The sequencing 2D gels are generated from serial, whole-tissue section cryostat recuts that contain abundant amounts of protein representing all cell types present in the tissue, including the dissected cell populations. Alignment of the diagnostic and sequencing 2D gels permits determination of proteins of interest for subsequent mass spectrometry or N-terminal sequence analysis. As an example, we were able to successfully visualize and align all 675 proteins present in the microdissected tumor cells from case #1 in the corresponding whole-tissue section 2D gel. Two of the tumor-specific alterations were selected for subsequent mass spectrometry and identified as cytokeratin 1 (overexpressed) and annexin I (underexpressed). Consistent with this result, cytokeratins and annexin I have been previously reported to be dysregulated in epithelial tumors [31-37].

One conclusion to be drawn from the present study is the importance of comparing matched normal and tumor cells from the same patient. Of the seven proteins found to be downregulated in the tumor, two were observed uniquely in the normal epithelium of case #1, and one was present uniquely in case #2. These proteins are "patient-specific" and would not have been identified if a single reference normal sample had been used as representative of the tumors. Therefore, direct intrapatient comparison of normal-tumor pairs is essential to ensure detection of all protein alterations that occur during tumorigenesis. Moreover, since little is known of the similarities and differences in protein profiles from person to person, it will be important in future proteomic studies to include interpatient analyses of both normal and tumor cells. These efforts may reveal patient-unique protein profiles related to cancer susceptibility and/or disease progression.

In summary, the data show that proteomic analysis of microdissected cell samples is feasible and can uniquely reveal tumor-specific alterations. These changes in protein expression reproducibly

track with the malignant phenotype and reflect changes that occur specifically in the epithelial cells and not in the surrounding cell types which could serve as sources of confounding contamination. Furthermore, the proteins that are found to be differentially expressed can be identified and their expression status validated. The approach and methods described may have widespread applicability to the study of normal cells and associated neoplasia.

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