Proteomics* in Bladder Cancer Research: Protein Profiling of Bladder Squamous Cell Carcinomas

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Bladder cancer comprises a broad spectrum of tumors that includes transitional cell carcinomas (TCCs), squamous cell carcinomas (SCCs), adenocarcinomas, small cell carcinomas, and leiomyomas (Pauli et al. 1983). TCCs are by far the most prevalent tumors and represent nearly 90% of all bladder cancers in the Western Hemisphere. SCCs, on the other hand, encompass a small percentage (2–3%) of all bladder lesions diagnosed in Europe and America, but are very frequent (80%) in areas of Africa and the Middle East where *Schistosoma haematobium*, a parasite that induces bladder SCCs in humans, is prevalent (El-Bolkainy 1983). SCCs often arise in patients who have a history of many years of chronic inflammation, keratinizing squamous metaplasia, and bladder stones. SCCs are highly malignant and the success of treatment relies heavily on early detection.

The histogenesis of SCCs is unclear, although these lesions may arise from extensive squamous differentiation of TCCs, i.e., carcinoma in situ (CIS) or high-grade papillary TCCs, and from neoplastic transformation — on the basis of squamous metaplasia — of the bladder urothelium (Figure 1) (Sakamoto et al. 1992, Østergaard et al. 1997). SCCs are composed of one cell type closely resembling keratinocytes both in morphology and protein expression profiles (Celis et al. 1996b, Østergaard et al. 1997; compare also Figures 1 and 3), and exhibit distinct squamous features such as “pearl” formation and keratohyalin bodies. Grading of these tumors is subjective and takes into consideration the degree of nuclear polymorphism, nuclear to cytoplasmic ratio, and chromatin clumping, as well as the number of mitotic cells (Friedell et al. 1983).

To achieve a more reliable and objective classification of bladder SCCs that may eventually pave the way for studies of the molecular mechanisms underlying progression, we are exploring the possibility of using proteome expression profiles of these lesions as fingerprints to identify tumor subtypes and to define their grade of atypia (Celis et al. 1996b, Østergaard et al. 1997). Proteins are frequently the functional molecules and, therefore, the most likely to reflect qualitative (expression of new proteins, changes in posttranslational modifications) and quantitative (up and down regulation, coregulated proteins) differences associated with various stages of cancer development. Genes may be present; they may be mutated, but they are not necessarily transcribed. Some messengers are transcribed but not translated, and the number of mRNA copies does not necessarily reflect the number of functional protein molecules (Anderson and Seilhamer 1997). Consequently, because of these uncertainties, focusing on the proteins eliminates many of the problems encountered at the nucleic acid level.

In addition to the above-mentioned studies, we are interested in assaying the urine of patients with SCCs for biomarkers that may be of value for identifying individuals at risk (Celis et al. 1996b, Rasmussen et al. 1996, Østergaard et al. 1997). The strategy for searching for such markers is based on a systematic analysis of the proteome expression profile of tumors, other cell types in the urinary tract, their secreted and/or externalized proteins, as well as the patient’s urine. The strategy is straightforward although its implementation requires a full knowledge of the protein composition of all cell types in the urinary tract.

Characterization of SCC Proteins Utilizing the Keratinocyte 2-D PAGE Database

To date, the identification and characterization of SCC proteins has been greatly facilitated by the fact that SCC cells exhibit similar protein expression profiles as keratinocytes, a cell type for which we have identified about 35% of the 3,500 proteins resolved by high-resolution 2-D gel electrophoresis (2-D PAGE) (Celis et al. 1995, 1996a). In addition, we have gathered a substantial amount of behavioral protein data (identity, cellular localization, regulatory properties, etc.) that has greatly speeded up the analysis of the tumors (Celis et al. 1996b, Østergaard et al. 1997). This information is currently stored in 2-D PAGE proteomic databases that are available through the Internet thanks to custom software developed by Protein Databases, Inc., now owned by Bio-Rad. Figure 1 shows the synthetic master (IEF = isoelectric focusing) 2-D PAGE image of noncultured human keratinocyte proteins as depicted on the Web (http://biobase.dk/cgi-bin/celis).

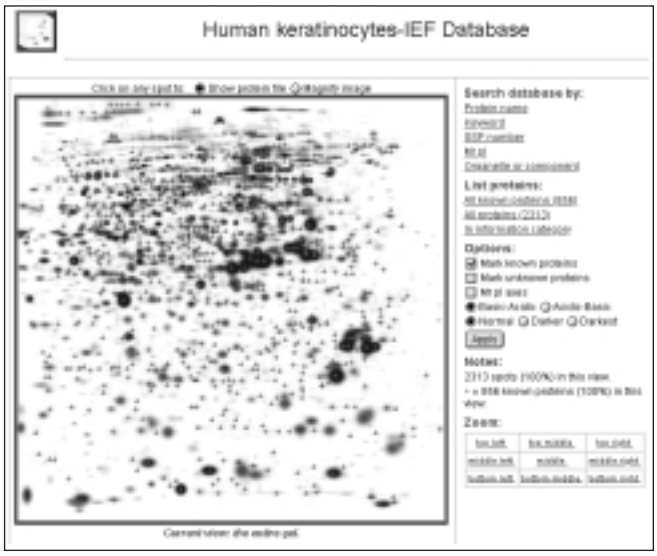

Fig. 1 (above left). Master synthetic image of human keratinocyte proteins separated by IEF (isoelectric focusing) 2-D PAGE as depicted on the Web. Proteins flagged with a red cross correspond to known proteins. By clicking on any spot it is possible to obtain a file containing information about the protein, as well as links to other databases and sites on the Internet.

Fig. 2 (above). File for the calcium-binding protein psoriasin. Only a few of the entries available on the Internet are shown.

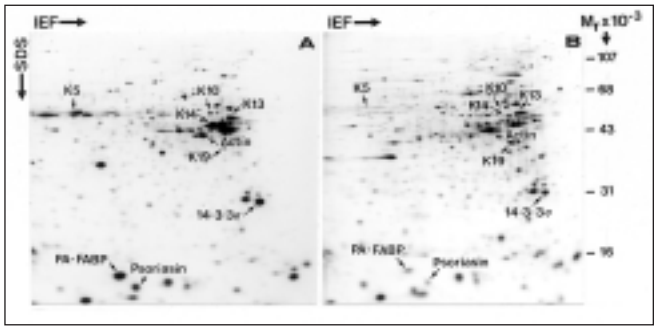

Fig. 3 (left). [35S]-methionine-labeled proteins from fresh SCCs separated by IEF 2-D PAGE. A, SCC 589-1; B, SCC 553-1. SCC 589-1 exhibits a higher degree of differentiation than tumor 553-1.

Proteins flagged with a red cross correspond to known polypeptides. About 1,200 polypeptides have been identified in this database (IEF and NEPHGE). Both microsequencing (Vandekerckhove and Rasmussen 1994, Nika and Aebersold 1994) and mass spectrometry (Wilm et al. 1996, Yates 1996, Pappin 1997) are instrumental for the identification of novel proteins, as they provide sequence information for molecular cloning of the corresponding mRNAs.

Information gathered on any given polypeptide, known or unknown, can be easily retrieved by clicking on the corresponding spot. A file containing all of the information entered for this particular protein, mostly obtained from experiments performed in our laboratory, appears as shown in Figure 2 (only a fraction of the file is shown). Files for known proteins contain links to public protein databases including a subset of MedLine, SWISS-PROT, and PDB. Other links include OMIM, GeneCards, Unigene, and other web sites such as CySPID, metabolic pathways, the cytokine explorer, histology images, etc. In the future, as new databases and related web sites become available, it will be possible to navigate through various databanks containing complementary information (nucleic acid and protein sequence, genome mapping, diseases, protein structure, posttranslational modifications, antibodies, signaling pathways, etc.). In particular, as the Human Genome Project progresses, 2-D PAGE databases are expected to annotate DNA sequences, and will be instrumental in linking protein and DNA sequencing with mapping information, offering a global approach to the study of cell regulation (Celis et al. 1990, 1996a). In addition, human proteomic databanks are expected to expedite drug discovery by pinpointing candidate drug targets on the basis of changes in the proteome expression profile of biopsies obtained from patients and controls (Celis et al. 1996a).

Currently, a great deal of effort is being invested in the development of similar 2-D PAGE databases of SCCs, TCCs, and urine (http://biobase.dk/cgi-bin/celis).

Identification of Protein Markers that Define the Degree of Differentiation of SCCs

So far, the analysis of fresh tumors labeled overnight with [35S]-methionine has revealed a few protein markers that are highly up-regulated in the more differentiated lesions. These include the calcium-binding protein psoriasin, the psoriasis associated fatty acid binding protein PA-FABP (FABP-5), galectin 7, the component of the *Ras-Raf* pathway 14-3-3 sigma (also known as stratifin), and keratins 5, 10, and 14 (Celis et al. 1996b, Østergaard et al. 1997 and references therein). Other markers useful in assessing the grade of differentiation and origin of the tumors correspond to keratins 13 and 19. In general, SCCs do not express keratins 7, 8, and 18, but many express abundant levels of keratin 19, which does not seem to be expressed by pure SCCs. Figure 3 shows the protein expression profiles of 2 SCC lesions that differ in their degree of differentiation, SCC 589-1 being the more differentiated one (Figure 3A).

It should be stressed that the above-mentioned markers are not expressed by all cells in a given tumor, a fact that emphasizes the need to perform immunohistochemical analysis in combination with 2-D PAGE in order to interpret the data. To decide which antibody should be included in the immunohistochemistry battery is a long process that requires first the analysis of the protein expression patterns of many tumors exhibiting various degrees of differentiation. Once major protein differences are found, the next step consists of identifying the polypeptides by using proteomic technology.

Identification of a Biomarker Externalized to the Urine by SCC-Bearing Patients

Proteome analysis of fresh SCCs as well as of patients' urine using 2-D PAGE and protein identification techniques has so far revealed a single biomarker, the calcium-binding and chemoattractant protein psoriasin (Madsen et al. 1991), which is synthesized by some differentiated cells in SCCs and that can be detected in the urine of SCC patients using immunoblotting in combination with enhanced chemi-luminescence detection (Celis et al. 1996b, Rasmussen et al. 1996, Østergaard et al. 1997). With the exception of urothelial tissue undergoing keratinizing and nonkeratinizing squamous metaplasia, this protein is not expressed in detectable amounts by any other cell type in the urinary tract (Celis et al. 1996b, Østergaard 1997). Psoriasin, alone or preferably in combination with other biomarkers, may prove valuable for the noninvasive follow-up of SCC patients, in particular males, as we have previously shown that the frequent presence of stratified squamous epithelia in the female trigone may lead to false positives (Celis et al. 1996b).

Identification of Premalignant Lesions

A major aim of our studies is to reveal premalignant lesions that may identify individuals at risk. This goal is being pursued systematically by analyzing the proteome expression profiles and immunofluorescent staining patterns of tumors and urothelial tissue from patients that have undergone cystectomy, that is, removal of the bladder due to invasive disease. Since bladder cancer is a field disease (Slaughter et al. 1953), that is, a large part of the bladder urothelium is at risk of developing disease, we surmised that small areas in the urothelium of patients with field disease may exhibit expression profiles and immuno-fluorescent staining patterns that closely resemble those observed in the invasive tumors. These lesions, if detected, may provide important clues about protein expression profiles and immunofluorescence patterns that may be associated with bad prognosis.

To illustrate the approach, Figure 4 shows immunofluorescent staining of cryostat sections of an SCC tumor (Figures 4B, E, H, K, and N) and urothelium with field disease (Figures 4C, F, I, L, and O) obtained from a patient with invasive disease. As depicted in the figure, the tumor does not stain with keratin 19 antibodies (Figure 4B), is BG3C8-antigen positive (Figure 4E) (Celis et al. 1996b), keratin 13 negative (Figure 4H), keratin 14 positive (Figure 4K), and psoriasin negative, with the exception of the pearls (Figure 4N). Similar analysis of the urothelium with field disease revealed a small area (indicated with large arrows) that stained weakly with the keratin 19 antibody (Figure 4C, indicated with an arrow) and that is BG3C8-antigen positive (Figure 4F), keratin 13 negative (Figure 4I), keratin 14 positive (Figure 4L), and psoriasin negative (Figure 4O). Other regions of the urothelium, one of which is indicated with small arrows, showed immunofluorescence patterns that were clearly different from those observed in the invasive tumor. We believe that a thorough analysis of the different antigen expression patterns detected in tumors and urothelium with field disease may prove instrumental in identifying those that may progress to invasive disease. As reference, we show in Figures 4A, D, G, J, and M immunofluorescence pictures of cryostat sections from normal urothelium obtained from another patient.

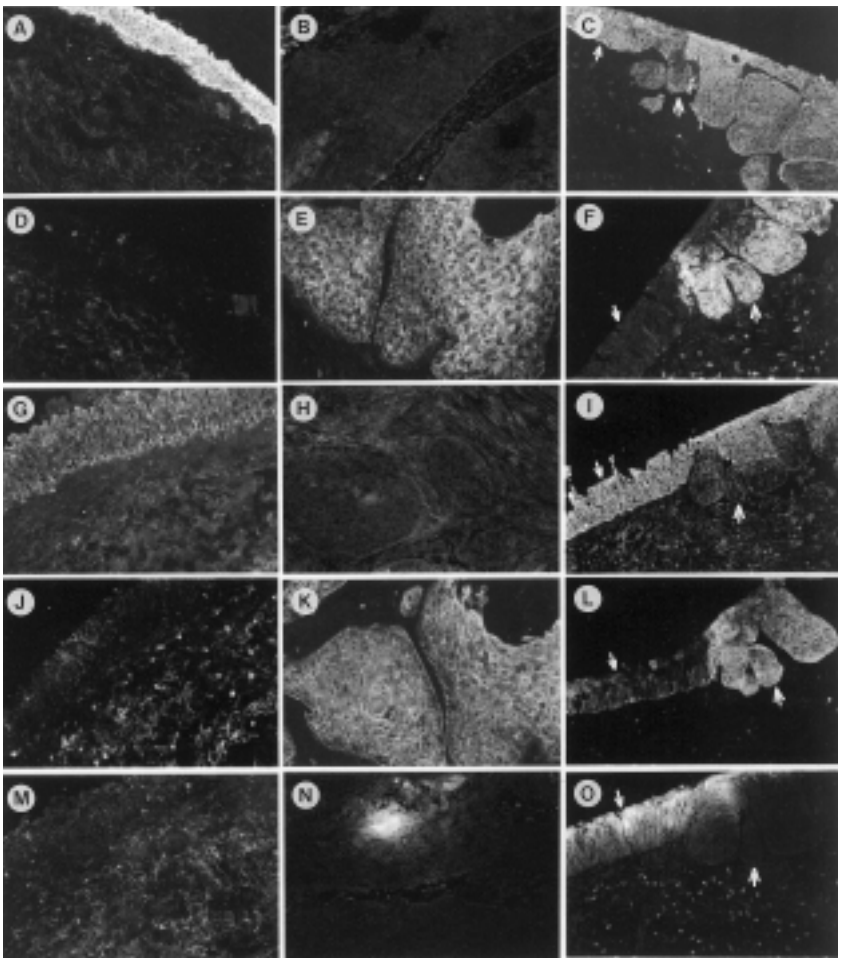

Fig. 4. Immunofluorescence pictures of formaldehyde-fixed cryostat sections of normal urothelium (A, D, G, J, and M), SCC 798-1 (B, E, H, K, and N), and urothelium 798-1 (C, F, I, L, and O) reacted with antibodies against keratin 19 (A, B, and C), the basal antigen BG3C8 (D, E, and F), keratin 13 (G, H, and I), keratin 14 (J, K, and L), and psoriasin (M, N, and O).

Perspectives

Clearly, state-of-the-art proteomic technology, in combination with immunofluorescence analysis of cryostat sections, offers a powerful approach for searching for protein biomarkers that may be associated with the various stages of tumor progression. The heterogeneity of the tissues and tumors poses obvious restrictions in the interpretation of the proteome data, although we believe these limitations will be overcome as additional markers become available that define the various stages of tumor progression. Currently, a great deal of effort is being devoted to quantitate the tumor protein profiles using the phosphor imager marketed by Bio-Rad, as it may be possible to rely on quantitative expression patterns to characterize particular stages in tumor development.

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* The term "proteome" was introduced by Wilkins et al. (1996). We consider proteomics as the global study of gene expression using a collection of techniques to resolve (high-resolution 2-D gel electrophoresis), quantitate (phosphor imager, special scanners), identify, and characterize proteins (microsequencing, mass spectrometry) as well as to store, communicate, and interlink protein and DNA sequence and mapping information (bioinformatics); see Anderson and Seilhamer (1997).

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