Proteomic Strategies to Reveal Tumor Heterogeneity among Urothelial Papillomas*

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Proteomics and immunohistochemistry were used to reveal tumor heterogeneity among urothelial papillomas (UPs) with the long term goal of predicting their biological potential in terms of outcome. First, we identified proteins that were deregulated in invasive fresh lesions as compared with normal urothelium, and thereafter we immunostained UPs with a panel of antibodies against some of the markers. Twenty-two major proteins showing variations of 2-fold or more in at least one-third of the invasive lesions were selected. Specific antibodies against several of the proteins were obtained, but only a few reacted positively in immunostaining. A panel consisting of antibodies against keratinocytes (CKs) 5, 13, 18, and 20 and markers of squamous metaplasia (CKs 7, 8, and 14) was used to probe normal urothelium and 30 UPs collected during a period of five years. Four UPs showed a normal phenotype, whereas the rest could be grouped in five major types that shared aberrant staining with the CK20 antibody. Type 1 heterogeneity ($n = 4$) showed preferred staining of the umbrella cells with the CK8 antibody. Type 2 ($n = 11$) was typified by the staining of the basal and intermediate layers with the CK20 antibody. Type 3 ($n = 7$) was characterized by the predominant staining of the basal cell layer with the CK5 antibody. Type 4 ($n = 1$) showed areas of CK7 negative cells, whereas type 5 ($n = 3$) showed loss of staining of the basal cells with the CK20. 29% of the patients experienced recurrences, but none progressed to invasive disease. Patients harboring phenotypic alterations in the basal cell compartment (types 3 and 5) showed the highest number of recurrences (4/7 and 2/3, respectively), and all type 3 lesions progressed to a higher degree of dedifferentiation. Even though a long term prospective study involving a larger sample size is required to assess the biological potential of these lesions, we believe that this approach will prove instrumental for revealing early phenotypic changes in different types of cancer. ***Molecular & Cellular Proteomics 1:269–279, 2002.***

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Received, November 23, 2001, and in revised form, March 21, 2002
Published, MCP Papers in Press, March 21, 2002, DOI 10.1074/mcp.M100031-MCP200

Bladder cancer is one of the most prevalent type of cancer in Denmark, with 11,509 patients in 1995 and more than 1000 new incidents every year (1). It comprises a broad spectrum of histological heterogeneous tumor types arising predominantly in the epithelium (urothelium) lining of the urinary bladder and the ureters. The urothelium is a multilayer epithelium containing polygonal basal cells that proliferate and differentiate to form the large umbrella-shaped cells of the superficial layer. The latter exhibit a unique trilaminar rigid membrane, termed the asymmetric unit membrane, that is in direct contact with the urine (2).

Tumor types of the urothelium include transitional cell carcinomas (TCCs),[1] squamous cell carcinomas, and adenocarcinomas, as well as other less frequent lesions (3). TCCs comprise more than 90% of the diagnosed bladder tumors in the Western hemisphere and are divided broadly into papillary (growing into the lumen of the bladder) and flat lesions that arise and progress through different genetic alterations (4). Bladder tumors are diagnosed routinely by cytoscopy, which give important information on multifocality, appearance, and size (5, 6). Cytoscopy is essential for resection and provides valuable material for pathological observation and research purposes. At present, pathological staging and grading are the most significant prognostic factors for treatment and outcome.

The biological relevance of low grade papillary tumors is underlined by the fact that about 70% of the bladder lesions are diagnosed as superficial (Ta, T1) at first presentation. These tumors have a high frequency of recurrence (>60%), and about 10–15% of them will progress to life-threatening malignancies (7, 8). Currently, it is not possible to assess with certainty the biological behavior of these tumors based on clinical or morphological criteria alone, and as a result it is urgent to identify early and accurate biomarkers that may predict recurrence, progression, and response to treatment. Moreover, it is important to distinguish those lesions that have no significant effect on the life expectancy, as all tumor-bearing patients are diagnosed with cancer, a fact that has

[1] The abbreviations used are: TCC, transitional cell carcinoma; IEF, isoelectric focusing; UP, urothelium papilloma; 2D, two-dimensional; A-FABP, adipocyte fatty acid-binding protein; PA-FABP, psoriatic-associated protein FABP; PCNA, proliferating cell nuclear antigen; HBSS, Hanks'-buffered saline solution; CK, keratinocytes.

practical and economic implications, as well as a profound psychological effect on the patient (9). To date, several prognostic markers have been identified; these include FDA-approved biomarkers (NMP22, fibrin/fibrinogen degradation product, and basement membrane components), blood group-related antigens (ABH, Lewis antigen), tumor-associated antigens (M344, 19A211, T138, DD23), proliferation antigens (Ki67 antibody, PCNA), oncogenes (c-Erb B2, Ras, c-Myc, mdm2), growth factors (epidermal growth factor, transforming growth factor-β, fibroblast growth factor, vascular endothelial growth factor), adhesion molecules (cadherins, integrins), cytokeratins (keratin 20), and cell cycle regulatory proteins such as p53, pRb, cyclins, p15, p16, and p21 (10–12). However, with very few exceptions these markers do not predict the biological behavior of low grade lesions. Today, it is becoming increasingly clear that multiple, rather than single markers, may be required to predict accurately prognosis and response to treatment.

In our laboratories we are applying gene expression profiling technologies in combination with immunohistochemistry to reveal bladder cancer heterogeneity with the long term aim of predicting the biological behavior of these lesions in terms of recurrence and progression (13–15). An important milestone of these studies has been the establishment of proteomic databases of various bladder tissue compartments (urothelium, TCCs, squamous cell carcinomas, muscle, connective tissue), as well as of urine and plasma (biobase.dk/cgi-bin/celis; see Refs. 16 and 17). The strategy for identifying tumor heterogeneity (18) encompasses a blind and systematic study of the proteome expression profiles of hundreds of fresh biopsy specimens from both normal and tumor origin. First, we identify major proteins that are expressed differentially in invasive lesions as compared with normal urothelium, and thereafter we use specific antibodies against these proteins to immunostain cryostat sections of tumors diagnosed as having the same stage and grade of atypia. Here we report on the analysis of 30 fresh Gr I, Ta, UPs collected at Skejby Hospital in Aarhus over a period of 5 years.

MATERIALS AND METHODS

Tumors—Tumors and other biopsies removed at Skejby Hospital, Aarhus, Denmark (informed consent was obtained from the patient) over a period of 5 years were placed immediately on ice and transported to the laboratory. Tumor pieces for cryostat sections were kept at −80 °C. Tumors were classified according to Bergkvist *et al*. (19). In this classification, Gr I, Ta lesions are defined as papillomas, that is a papillary tumor with a thin fibrovascular stroma and a normal looking urothelium. This corresponds to the World Health Organization group papillary lesions of low malignant potential. The Scientific and Ethical Committee of Aarhus County approved the project.

Labeling of Tumors with [^{35}S]Methionine—Tumors clean of clots and contaminating tissue were minced in small pieces with the aid of a scalpel and were labeled with [^{35}S]methionine for 14–16 h in a 10-ml sterile plastic conical tube containing 0.2 ml of modified Eagle's medium lacking methionine, 2% dialyzed (against 0.95% NaCl) fetal calf serum, and 100 μCi of [^{35}S]methionine (SJ204; Amersham Biosciences). At the end of the labeling period the medium was aspirated, and the pieces were dissolved in 0.3 to 0.4 ml of lysis solution with the aid of a 1-ml plastic pipette (20). Samples were stored at −20 °C until use.

Labeling of Normal Random Biopsies with [^{35}S]Methionine—The urothelium from random biopsies diagnosed as normal was dissected with the aid of a scalpel, labeled with [^{35}S]methionine as described above, and processed for 2D-PAGE (21). Samples were stored at −20 °C until use.

Two-dimensional PAGE—2D-PAGE was performed essentially as described by Celis *et al*. (21). Gels were stained with silver nitrate (22), dried and exposed to x-ray films as described previously (21).

Protein Identification—Proteins were identified using a combination of procedures that included mass spectrometry, namely matrix-assisted laser desorption ionization time-of-flight, Biflex (Bruker) (22), 2D-PAGE Western immunoblotting (23), and comparison with the master 2D gel images of human keratinocytes and TCC proteins (24) (http:/biobase.dk/cgi-bin/celis).

Antibodies—Rabbit polyclonal antibodies against CK5, CK13, A-FABP, PA-FABP, maspin, 14–3-3σ, and hsp28. MRP14 and PCNA were prepared in our laboratory. Monoclonal antibodies against CKs 7, 8, and 18 were purchased from ICCN/Cappel. The monoclonal antibody against CK20 was purchased from Cymbus, and the CK14 antibody was purchased from AH Diagnostics. All antibodies were screened carefully for specificity by 2D-PAGE Western immunoblotting (IEF, non-equilibrium pH gradient electrophoresis) of different cell types and human tissues, including TCCs (23). In addition, they were screened using a battery of human tissues that either expressed or lacked the protein in question.

Indirect Immunofluorescence—Tumors and random biopsies frozen in liquid nitrogen were sectioned on a Reichert-Jung cryostat. 8-μm sections placed in coverslips were washed three times with Hanks'-buffered saline solution (HBSS) and treated for 10 min with 3.6% formaldehyde (18). After washing extensively with HBSS, the coverslips were covered with 20 μl of the primary antibody and incubated for 45 min at 37 °C in a humid environment. The coverslips were washed several times with HBSS and covered with 20 μl of rhodamine-conjugated secondary antibody (diluted 1:50 in HBSS). After 45 min of incubation at 37 °C in a humid environment, the coverslips were washed thoroughly with HBSS and mounted in fluorescence mounting medium (DAKO). Observations were made on a Leica photomicroscope equipped with epifluorescence.

RESULTS

Identification of Major Proteins Highly Deregulated in Invasive TCCs

Fresh tumor and normal biopsies collected during a period of 5 years were analyzed by 2D-PAGE and autoradiography as described under "Materials and Methods." Biopsies were labeled with [^{35}S]methionine as this improved by at least one order of magnitude the number of polypeptides detected as compared with silver staining, and in addition, avoided the problem associated with the presence of serum proteins, particularly in the case of invasive tumors. Of the several hundred specimens that were received, 122 corresponded to Gr III, T2–4 TCCs at first presentation. These tumors were the most numerous among the invasive lesions and were chosen to generate the markers as they were expected to harbor proteome alterations associated with recurrence and progression.

Only biopsy specimens from invasive tumors exhibiting minor contamination with connective and/or muscle tissue as

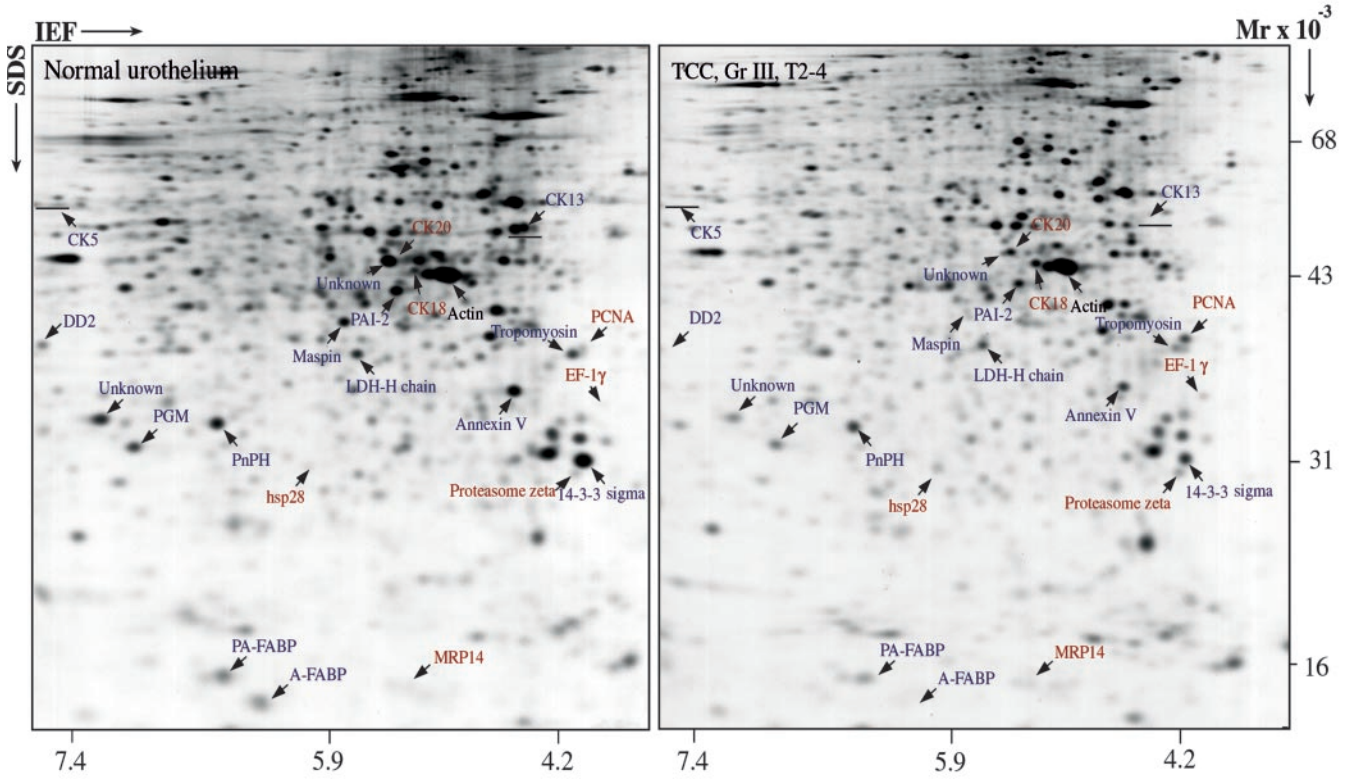

FIG. 1. **Proteins highly deregulated in invasive TCCs.** [^{35}S]Methionine-labeled proteins synthesized by normal urothelium and an invasive TCC (Gr III, T2–4) were separated by 2D-PAGE (IEF) and visualized by autoradiography. Polypeptides indicated with *red* are up-regulated in the tumor whereas those indicated in *blue* are down-regulated. The position of actin is indicated for reference.

judged by the expression of vimentin and desmin using immunostaining and 2D gel analysis (13) and that yielded fair separation profiles (30 lesions) were selected for comparison with normal urothelium. A normalized base line of protein expression of "normal" urothelium was attained by visual analysis of 116 random biopsies classified as normal by the pathologist irrespective of whether they were derived from patients bearing low grade or invasive TCCs. Only proteins whose expression was highly deregulated (2-fold or more) in the invasive lesions (see Fig. 1 and Table I) were used as reference. These included A-FABP, PS-FABP, PCNA, CK13, 14–3-3σ, and MRP14. With the exception of A-FABP and MRP14, which showed deregulation in 20.6 and 14.7% of the random biopsies, respectively, all other markers showed fluctuations in less than 3% of the biopsies. It should be stressed that in the case of A-FABP, more than 50% of the random biopsies showing strong down-regulation were derived from patients bearing invasive tumors.

Fig. 1 shows representative proteome expression profiles of fresh biopsies from normal urothelium and an invasive TCC (Gr III, T2–4). Even though the overall expression profiles are remarkably similar, the level of several major polypeptides was altered highly in the invasive lesions. Deregulated polypeptides that showed variations of 2-fold or more in at least 40% of the invasive lesions, as determined by visual inspection of the 2D gels, were selected. The cutoff value of 33% was chosen to include CK20 as the expression of this differentiation marker has been shown to be aberrant in recurrent papillary tumors (9). In reality, however, most of the selected markers were deregulated in more than 50% of the lesions (Table I). Deregulated polypeptides are indicated in red (up-regulated) and blue (down-regulated) in Fig. 1. Their coordinates (apparent M_r and pI) in the TCC database, the frequency at which the change occurred in the invasive lesions, and the putative functions are listed in Table I. Up-regulated proteins included elongation factor 1γ, hsp28, CKs 18 and 20, PCNA, the proteasome ζ subunit, and MRP14. Down-regulated proteins comprised the A-FABP, annexin V, the high affinity bile-binding protein (DD2), CKs 5 and 13, lactate dehydrogenase H chain, maspin, plasminogen activator inhibitor 2, PA-FABP, phosphoglycerate kinase, purine nucleoside phosphorylase, a tropomyosin isoform, 14–3-3σ, (stratifin), and two unknown proteins recorded in the TCC 2D-PAGE database. Changes in the levels of CK5, hsp28, and MRP14 are not observed in the biopsy pairs depicted in Fig. 1, but representative areas of gels from other normal and invasive tumor specimens are shown in Fig. 2.

Antibodies against the Deregulated Protein Markers

Antibodies against 13 of the putative protein markers were raised in rabbits (hsp28, PCNA, MRP14, A-FABP, PA-FABP,

TABLE I
Polypeptides deregulated in invasive TCCs

Identity	Molecular mass (*kDa*)	pI	Method of identification	Function	Altered expression incidence[a]	Antibodies and specificity[b]	Immunofluorescence in fixed cryostat sections
				Up-regulated polypeptides			
EF 1-γ	33.0	4.3	Mass spectrometry, comparison with the keratinocyte 2D-PAGE database	Protein synthesis	9/15 (60%)		
hsp28	25.5	6.0	Mass spectrometry, comparison with the keratinocyte 2D-PAGE database	Stress response: heat shock, cell differentiation	19/24 (79%)	Rabbit polyclonal[c] Specific	Negative
CK18	43.6	5.3	Mass spectrometry, 2D-PAGE immunoblotting	Cytoskeleton assembly	13/23 (57%)	Monoclonal[d] Specific	Positive
CK20	45.7	5.6	Mass spectrometry, 2D-PAGE immunoblotting	Cytoskeleton assembly	6/18 (33%)	Monoclonal[d] Specific	Positive
PCNA	35.8	4.4	Mass spectrometry, 2D-PAGE immunoblotting	Cell cycle and differentiation	16/21 (76%)	Rabbit polyclonal[c] Specific	Negative
Proteasome ζ	28.2	4.4	Mass spectrometry	Protein turnover	11/21 (52%)		
MRP14	11.2	5.6	Mass spectrometry, comparison with the keratinocyte 2D-PAGE database	Calcium-modulated metabolism: cell migration	14/17 (82%)	Rabbit polyclonal[c] Specific	Negative
				Down-regulated polypeptides			
A-FABP	11.7	6.2	Mass spectrometry, 2D-PAGE immunoblotting	Cell cycle and differentiation: fatty acid transport	25/25 (100%)	Rabbit polyclonal[c] Specific	Negative
Annexin V	35.5	4.8	Mass spectrometry, 2D-PAGE immunoblotting	Calcium-modulated metabolism: Ca^{+} and phospholipid-binding protein	17/23 (74%)	Rabbit polyclonal[e] Specific	Negative
High affinity bile-binding protein (DD2)	36.7	7.5	Edman degradation	Member of oxidoreductase gene family: multifunctional protein, involved in bile acid transport	9/17 (53%)		
CK5	57.8	7.8	2D-PAGE immunoblotting	Cytoskeleton assembly	12/17 (71%)	Rabbit polyclonal[c] Slight cross-reactivity with CKs 7 and 8	Positive
CK13	52.2	4.8	2D-PAGE immunoblotting	Cytoskeleton assembly	20/26 (77%)	Rabbit polyclonal[c] Specific	Positive
Lactate dehydrogenase H chain	35.5	5.7	Mass spectrometry	Energy metabolism: mitochondrial respiration	17/22 (77%)		
Maspin	38.2	5.8	Mass spectrometry	Cell cycle and differentiation: tumor supression	17/22 (77%)	Rabbit polyclonal[c] Specific	Negative
PAI-2	41.3	5.5	Mass spectrometry	Member of serpine protease-inhibitor family: extracellular proteolysis	16/25 (64%)	Rabbit polyclonal[e] Specific	Negative
PA-FABP	12.4	6.3	Mass spectrometry 2D-PAGE immunoblotting	Cell cycle and differentiation: fatty acid transport	22/23 (96%)	Rabbit polyclonal[c] Specific	Negative
PGM	31.0	6.8	Mass spectrometry	Energy metabolism	20/22 (91%)		
PnPH	31.9	6.4	Mass spectrometry	Cell cycle and differentiation: DNA metabolism	19/22 (86%)		
Tropomyosin	33.7	4.4	Mass spectrometry, comparison with the keratinocyte 2D-PAGE database	Actin filament assembly	15/21 (71%)		
14-3-3 σ	30.0	4.4	Mass spectrometry, comparison with the keratinocyte 2D-PAGE database	Cell cycle and differentiation: mitogenic signal transduction	17/20 (85%)	Rabbit polyclonal[c] Cross-reactivity with other family members	Negative
Unknown[f]	45.6	5.6			9/17 (53%)		
Unknown[f]	31.7	6.9			11/16 (69%)		

[a] Based on the 2D gel analysis of 30 invasive TCCs (Gr III, T2-4) labeled with [^{35}S]methionine. In several cases the entire gel image could not be used for analysis because of focusing problems in different areas of the gels. Only major proteins exhibiting more than a 2-fold change are listed.

[b] Determined by 2D gel Western blotting.

[c] Prepared in our laboratory.

[d] Available commercially.

[e] Obtained from colleagues.

[f] Unknown in the TCC 2D-PAGE database.

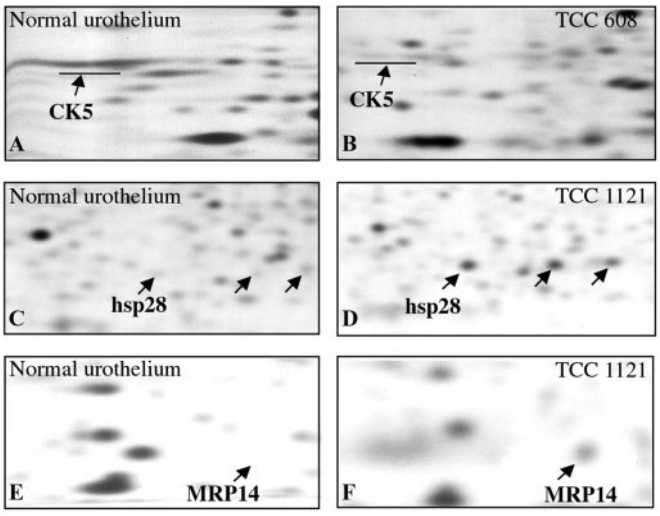

FIG. 2. **Levels of CK5, hsp28, and MRP14 in normal urothelium and invasive TCCs.** [^{35}S]Methionine-labeled proteins synthesized by normal urothelium and invasive TCCs (Gr III, T2–4) were separated by 2D-PAGE (IEF) and visualized by autoradiography. *A*, *C*, and *E*, normal urothelium; *B*, *D*, and *F*, invasive lesions. Only a fraction of the IEF 2D-gels are shown.

maspin, 14–3-3σ, and CKs 5 and 13), purchased from commercial sources (CKs 18 and 20), or procured from colleagues (annexin V, plasminogen activator inhibitor 2). With the exception of two antibodies that showed slight cross-reactivity with other family members (14–3-3σ and CK5; see Fig. 3, *F* and *G*), the rest were highly specific as judged by 2D-PAGE immunoblotting using both isoelectrofocusing (see Fig. 3; blots of PA-FABP and annexin V antibodies are not shown) and nonequilibrium pH gradient electrophoresis (see "Materials and Methods"). Differential expression of the markers was confirmed by 2D-PAGE immunoblotting using representative pairs of normal and invasive lesions (not shown). Only antibodies against CKs 5, 13, 18, and 20 reacted positively with methanol or formaldehyde-fixed cryostat sections and were included in the battery of antibodies to immunostain UPs.

Identification of Tumor Heterogeneity among Gr I, Ta Urothelial Papillomas

The study group consisted of 30 patients that were diagnosed with Gr I, Ta UPs at first presentation over a period of 5 years. Clinical features, as well as recurrences, are given in Table II. Patient age at diagnosis ranged from 27 to 88 years old, and the male to female ratio was 2:1.

Frozen cryostat sections of the 30 UPs fixed in formaldehyde were immunostained with the panel of antibodies described above. The battery was supplemented with antibodies specific for CKs 7, 8, and 14 (Fig. 3), and we and others (18, 25–27) have shown that these proteins are markers for squamous differentiation, a rather common feature of invasive lesions (26, 27).[2] Indeed, the levels of these proteins were deregulated in a significant number of invasive lesions (results not shown).

With the exception of four UPs that displayed identical staining patterns as normal urothelium, the rest could be grouped in five major types that, with the exception of type 1, shared aberrant staining with the CK20 antibody, a predictor of tumor recurrence (9). UPs exhibited only one major type of tumor heterogeneity, but the extent of the heterogeneity was variable and in most cases included variants.

The staining phenotypes of normal urothelium reacted with the panel of antibodies are presented in Fig. 4. Antibodies against CKs 20 (Fig. 4*A*) and 18 (Fig. 4*B*) stained the superficial umbrella cells, whereas the probes against CKs 5 (Fig. 4*C*), 7 (Fig. 4*D*), and 8 (Fig 4*E*) reacted with basal, intermediate, and superficial layers in a homogenous fashion. No staining of the umbrella cells was observed with the CK13 antibody, which stained the basal and intermediate layers (Fig. 4*F*) (18, 27), and as expected, none of the cell layers reacted with the CK14 antibody (Fig. 4*G*). For reference, a hematoxylin staining of normal urothelium is included in Fig. 4*H*. Because of space limitations only major staining differences between the subtypes and normal urothelium are highlighted below (see also Table III).

Type 1 heterogeneity was found in four UPs and was characterized by the predominant staining of the umbrella cells with the CK8 antibody (Fig. 5, *A*, compare with CK18 staining in *B*). All urothelial cell layers stained throughout with the CK20 antibody (not shown), although the umbrella cells stained stronger. 2D-PAGE analysis of total protein extracts from these tumors suggested that the increased staining of the superficial cells with the CK8 antibody is most likely not because of increased synthesis of the protein or because of major post-translational modification(s) that affect its charge (compare Fig. 6, *A* and *B*).

Type 2 heterogeneity was observed in 11 UPs and was typified by the abnormal expression of CK20 in the basal and intermediate layers (Fig 5*C*). Only very few umbrella cells stained with this antibody. Variants within this type included tumors that exhibited either scattered cells expressing CK18 in non-umbrella cells (type 2B, Fig. 5*D*) or areas with CK5 negative cells (type 2C, not shown).

Type 3 heterogeneity was detected in seven UPs and was characterized by the preferred staining of the basal cell layer with the CK5 antibody, as well as by the abnormal staining with the CK20 antibody (type 3A; see Fig. 5, *E* and *F*). In some tumors the staining was accompanied by scattered cells expressing CK18 (type 3B; not shown) and, in addition, by basal expression of CK14. The expression of CK14 was confirmed by 2D-PAGE (Fig. 6*C*).

Type 4 heterogeneity was detected in one UP that showed large areas of CK7 negative cells (Fig. 5*G*), a fact that was confirmed by 2D-PAGE analysis of total protein extracts (Fig. 6*D*). The lesion stained with the CK8 antibody but not with the CK14 probe. Even though the pattern was observed in only one tumor we classified it in a separate group as loss of CK7

[2] Unpublished results.

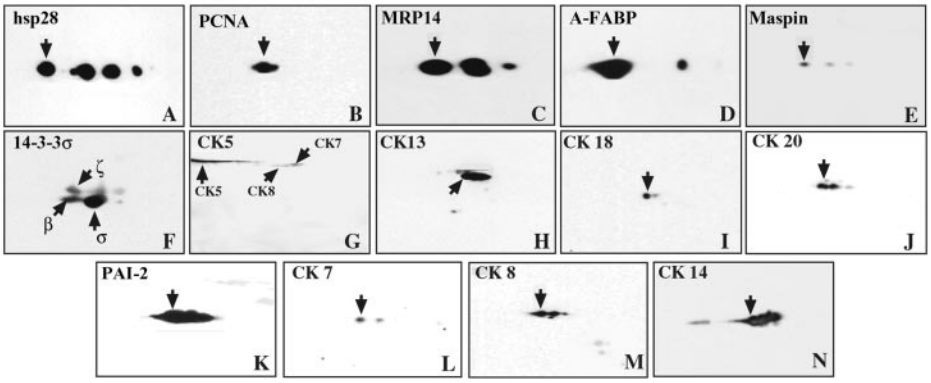

FIG. 3. **Antibody specificity as determined by 2D-PAGE Western immunoblotting (IEF) of total cellular proteins from normal human keratinocytes (*A–F*) and TCCs (*G–N*).** Blots were developed using the enhanced chemiluminescence procedure.

staining was observed in a significant number of invasive TCCs, as well as in some areas of the urothelium of patients with invasive disease.

Type 5 heterogeneity was observed in three UPs and was characterized by the abnormal expression of CK20, in particular by the lack of staining of the basal cell layer (Fig. 5*I*). One of the UPs displayed, in addition, CK14 in the basal layer (type 5B; see Fig. 5*J*).

Twenty-nine percent of the patients experienced recurrences during the course of the study, but to date none progressed to invasive disease. Patients with UPs harboring types 3 and 5 heterogeneities showed the highest number of recurrences, 4/7 and 2/3, respectively. Interestingly, all recurrent type 3 lesions progressed to a higher degree of dedifferentiation resulting in a higher histological grade (Gr II, Ta). No recurrences have been observed so far for the UPs that exhibited normal immunostaining with the panel of antibodies. It should be stressed, however, that some patients were recruited late in the course of the study, and as a result the recurrence data are limited.

DISCUSSION

To be able to predict which patients with UPs will develop recurrences and/or progress to invasive disease is one of the major challenges that we face today in bladder cancer research. Morphological parameters alone are not able to discern the biological potential and behavior of these tumors, and hence it is imperative to identify markers that may predict patient outcome. Recently, Southgate and colleagues (9) showed that papillary lesions displaying normal distribution of CK20 may not recur, whereas those exhibiting aberrant expression of this keratin, that is expression in all cell layers, had a higher frequency of recurrence. These results have been challenged recently by Alsheikh *et al*. (28), who compared the WHO/ISUP classification (29, 30) and CK20 expression in predicting the behavior of low grade papillary urothelial tumors. In this study, papillary neoplasms of low malignant potential had a lower recurrence rate than low grade papillary TCCs, and low grade urothelial tumors exhibiting normal CK20 expression pattern recurred less frequently than lesions with an aberrant pattern of CK20 staining. Neither of these differences was significant statistically, however, and recurrences were observed in 20% of patients whose tumors were both classified as papillary neoplasms of low malignant potential and showed normal CK20 immunostaining. The results presented here cannot be compared directly with those described above, because the number of tumors showing normal patterns of CK20 was small, and the recurrence data were sparse. In general, however, they support the contention that aberrant immunostaining of CK20 is an important phenotypic characteristic associated with some non-invasive papillary lesions.

Our approach for identifying tumor heterogeneity among UPs relied on a combination of proteomic technologies and immunohistochemistry and encompassed a blinded and systematic study of the proteome expression profiles of hundreds of fresh biopsy specimens of both normal and tumor origin. Invasive TCCs (Gr III, T2–4) rather than UPs were chosen to generate the putative protein markers as these lesions were expected to harbor proteome alterations associated with recurrence and progression that may be impossible to identify at an early stage. We surmised that areas of urothelial heterogeneity may comprise only a small proportion of the UPs, and as a result neither down- nor up-regulated protein markers may be detected under these conditions.

Although the size of the prospective study group was limited by the necessity of using fresh biopsy material, we were able to identify several types of tumor heterogeneity among 30 patients diagnosed with Gr I, Ta UPs at first presentation over a period of 5 years. The nature of the heterogeneity was defined utilizing only a limited number of proteins whose expression was deregulated by 2-fold or more and for which we were able to obtain specific antibodies that could be used with confidence in immunohistochemistry. This panel was supplemented with antibodies against markers of squamous metaplasia (CKs 7, 8, and 14) as these lesions are a rather common feature of invasive TCCs (26, 27).[3] Low abundance proteins were not targeted here as this study was intended as a proof of concept rather than as a comprehensive analysis of all possible protein markers. Certainly, future work must take

[3] Unpublished observations.

TABLE II
Urotelial papillomas (Gr I, Ta)

Case	Gender[a]	Age	Number of tumors and date at first presentation	Grade and stage	Type of tumor heterogeneity	Recurrences to date	Pathology description
519-1	M	65	1-12/10/95	Gr I, Ta	None detected[b]	None	Inverted papilloma
685-1	F	58	1-21/01/97	Gr I, Ta	None detected[b]	None	Papilloma
932-1	F	72	5/6-07/07/98	Gr I, Ta	None detected[b]	None	Papilloma
1080-1	M	68	1-21/04/99	Gr I, Ta	None detected[b]	None	Papilloma
521-1	M	27	1-13/10/95	Gr I, Ta	1	14/10/96 (Gr I, Ta) 14/09/97 (Gr I, Ta) 23/08/99 (Gr I, Ta)	Papilloma
681-1	F	50	1-07/01/97	Gr I, Ta	1	None	Papilloma
929-1	F	70	3-30/06/98	Gr I, Ta-2 Gr II, Ta-1	1	None	Papilloma
1084-1	M	58	1-04/05/99	Gr I, Ta	1	None	Papilloma
440-1	F	64	1-02/05/97	Gr I, Ta	2A	None	Papilloma
714-1	M	51	1-05/03/97	Gr I, Ta	2A	None	Papilloma
866-1	F	53	1-12/03/98	Gr I, Ta	2A	None	Papilloma
914-1	M	86	1-15/06/98	Gr I, Ta	2A	None	Papilloma
916-1	F	51	1-16/06/98	Gr I, Ta	2A	16/10/98 (Gr I, Ta) 22/02/99 (Gr I, Ta) 15/06/99 (Gr I, Ta) 07/10/99 (Gr I, Ta) 18/09/00 (Gr I, Ta) 23/01/01 (Gr I, Ta) 13/08/01 (Gr I, Ta)	Papilloma
1206-1	M	62	1-08/03/00	Gr I, Ta	2A	None	Papilloma
676-1	M	86	1-18/12/96	Gr I, Ta	2B	None	Papilloma
860-1	F	65	1-18/02/98	Gr I, Ta	2B	08/03/00 (Gr II, Ta)	Papilloma
954-1	M	74	1-01/09/98	Gr I, Ta	2B	None	Papilloma
1012-1	M	73	1-04/12/98	Gr I, Ta	2B	None	Papilloma
957-1	M	75	1-01/09/98	Gr I, Ta	2C	13/03/00 (Gr I, Ta) 09/10/00 (Gr II, Ta) 09/01/02 (Gr I, Ta)-4	Papilloma
793-1	M	67	1-06/08/97	Gr I, Ta	3A	07/09/98 (Gr II, Ta) 26/06/01 (Gr II, Ta)	Papilloma
972-1	M	82	1-02/10/98	Gr I, Ta	3A	13/04/99 (Gr II, Ta)	Papilloma
483-1	F	76	1-25/08/95	Gr I, Ta	3B	None	Papilloma
844-1	M	88	1-05/01/98	Gr I, Ta	3B	None	Papilloma
1246-1	M	81	1-10/05/00	Gr I, Ta	3B	None	Papilloma
703-1	M	79	1-12/02/97	Gr I, Ta	3C	17/08/98 (Gr II, Ta) 06/04/99 (Gr II, Ta)	Papilloma
1250-1	F	65	1-26/07/00	Gr I, Ta	3C	28/02/01 (Gr II, Ta) 03/09/01 (Gr I, ND)[c]	Papilloma
841-1	M	31	1-19/12/97	Gr I, Ta	4	None	Papilloma
493-1	M	77	1-08/09/95	Gr I, Ta	5A	23/09/96 (Gr I, T1) 07/02/97 (Gr I, Ta) 27/06/97 (Gr I, Ta)	Papilloma
755-1	M	48	1-16/05/97	Gr I, Ta	5A	None	Papilloma
898-1	M	78	3-28/04/98	Gr I, Ta-2 Gr II, Ta-1	5B	23/03/99 (Gr II, Ta) 28/09/99 (Gr I, Ta)	Papilloma

[a] F, female; M, male.
[b] As determined by staining with the panel of antibodies.
[c] Stage is not determined.

into consideration low abundance components as tumor cells may express 6000–7000 primary translation products plus their post-transitionally modified variants (31), many of which may be expressed at levels that escape detection with the current technology. Identification of differentially expressed proteins among low abundance polypeptides will not be an easy task as it will require the use of metabolic labeling with [^{35}S]methionine, enrichment techniques (microdissection (32, 33), subcellular fractionation (34), and purification of specific complexes (35)), as well as development of reliable, high

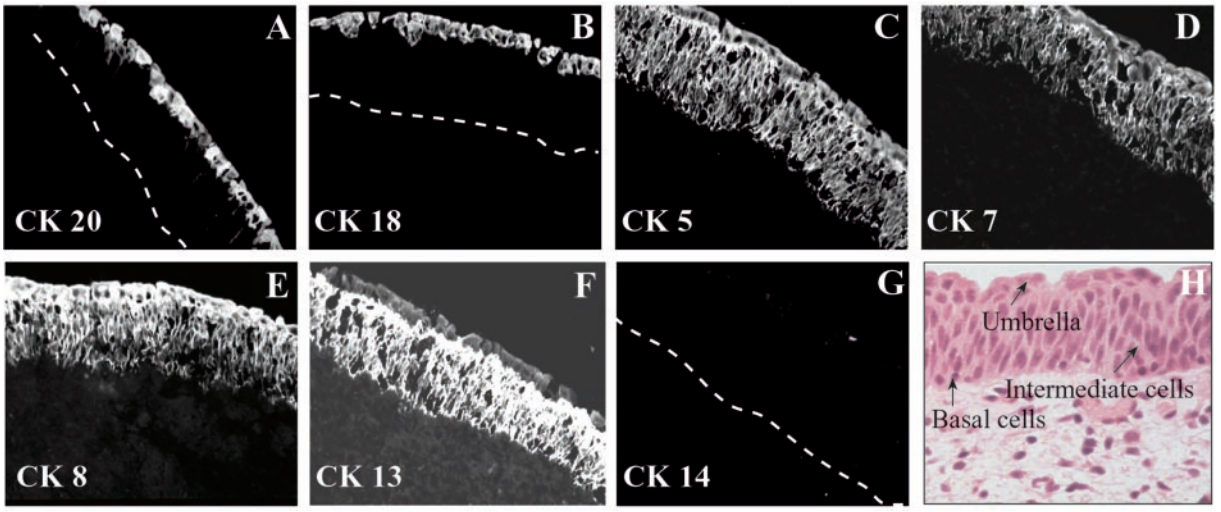

FIG. 4. **Immunofluorescence staining of formaldehyde-fixed cryostat sections from normal urothelium reacted with the panel of antibodies against keratins (*A–G*) and hematoxylin staining of normal urothelium (*H*).**

TABLE III
Antigen expression in normal urothelium and urothelial papillomas

For simplicity, only staining patterns that differ from those observed in normal urothelium are indicated.

Tissue	CK 20	CK 18	CK 5	CK 7	CK 8	CK 13	CK 14
Normal urothelium	Umbrella cells	Umbrella cells	All layers	All layers	All layers	Intermediate and basal cells	Negative
				Tissue Heterogeneity			
Type 1					Stronger staining of umbrella cells		
Type 2A	Intermediate and basal cells						
Type 2B	Intermediate and basal cells	Some scattered positive cells					
Type 2C	Intermediate and basal cells		Areas with negative cells				
Type 3A	Intermediate and basal cells		Stronger staining of basal cells				
Type 3B	Intermediate and basal cells	Some scattered positive cells	Stronger staining of basal cells				
Type 3C	Intermediate and basal cells	Some scattered positive cells	Stronger staining of basal cells				Some positive cells
Type 4	Intermediate and basal cells			Areas with many negative cells			
Type 5A	Intermediate cells; no staining of basal cells	No staining of umbrella cells					
Type 5B	Intermediate cells; no staining of basal cells	No staining of umbrella cells					Some basal cells positive

throughput quantitation procedures. Even if the latter requirements are overcome, we still need to address the problems associated with raising specific antibodies as the results presented here have clearly highlighted the limitations we face today regarding preparation, specificity, and reactivity of antibodies. Usually, one searches for heterogeneity that is evident only in very few cells, and as a result one must be confident that these cells express the antigen in question rather than an unknown cross-reactivity.

Of the 30 UPs analyzed by immunofluorescence using the panel of antibodies only four displayed identical staining patterns as normal urothelium. The rest could be grouped in five major types that, with the exception of type 1, shared aberrant staining with the CK20 antibody. As expected, the size of the areas showing heterogeneity varied from UP to UP, but most tumors exhibited only one major type of tumor heterogeneity. Main features of the different types of tumor heterogeneity are discussed below.

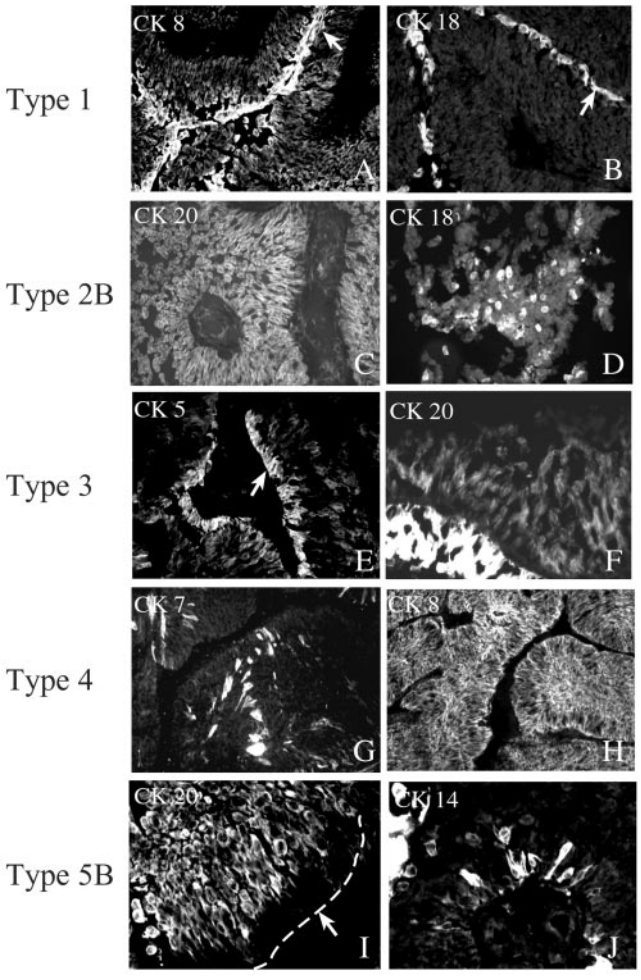

FIG. 5. **Tumor heterogeneity types.** Immunofluorescence staining of formaldehyde-fixed cryostat sections from selected UPs showing various types of tumor heterogeneity is shown.

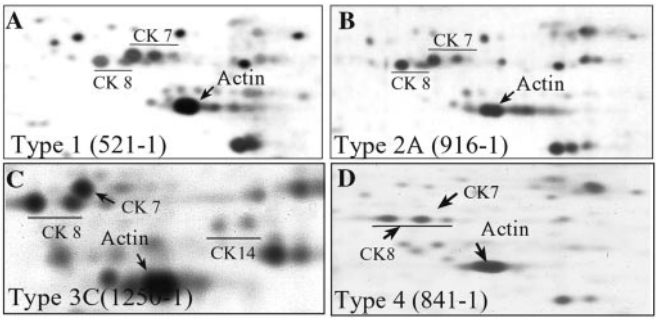

FIG. 6. **Expression of CKs 8, 14, and 7 in Gr I, Ta in selected UPs.** Proteins were separated by 2D-PAGE (IEF). *A* and *B*, silver-stained proteins from UPs 521–1 (type 1) and 916–1 (type 2A), respectively. *C* and *D*, [^{35}S]methionine-labeled proteins from UP 1250–1 (type 3C) and UP 841–1 (subtype 4), respectively. Only relevant areas of the 2D gels are shown.

Type 1 Heterogeneity

This type of heterogeneity ($n = 4$) was characterized by the stronger expression of CK8 in the superficial umbrella cells. This phenotype was not accompanied by changes in the expression levels of primary translation product or their modified variants, suggesting that the increased immunoreactivity of the umbrella cells is most likely because of epitope unmasking/masking phenomena that may be related to filament organization (26, 36). Differential staining of the urothelium has been observed using different CK8 antibodies (27), but it has not been reported in the case of paired tumor biopsies that have been stained with the same antibody. It should be emphasized that the CK8 antibody was included in the panel mainly because the levels of this protein decrease in squamous metaplasia (18). Evidently, type 1 heterogeneity is not related to squamous differentiation as none of the four UPs expressed CK14 (also confirmed by 2D gel profiling), a marker that can distinguish squamous differentiation even before it becomes evident by morphological criteria. In addition, we observed normal staining with the CK7 antibody in all the lesions showing this type of heterogeneity. Recently, we reported changes in the levels of CK8 in some invasive TCCs because of protein degradation (14), and we cannot exclude the possibility that this mechanism may play a role in the generation of type 3 heterogeneity.

Type 2 Heterogeneity

This type of heterogeneity was the most common ($n = 11$) and was characterized by the expression of CK20 in the basal and suprabasal layers as originally described by Harnden *et al*. (9). The levels of CK20 could not be determined by 2D-PAGE as the protein migrated very closely to a major labeled unknown protein that is down-regulated in invasive lesions (unknown protein close to CK20 in Fig. 1). Variants within type 2 heterogeneity included lesions expressing 1) focus of CK18 positive non-superficial cells, and 2) areas with CK5 negative staining. The latter changes could not be confirmed by 2D-PAGE analysis of UPs as the number of cells showing the phenotype was too small.

Type 3 Heterogeneity

This type of lesion ($n = 7$) was characterized by the stronger immunostaining of the basal cell layer with the CK5 polyclonal antibody. The antibody showed a weak cross-reactivity with CKs 7 and 8, but this did not interfere with the staining patterns. There is data in the literature, however, indicating that CK5 is expressed basically in normal urothelium as judge by immunostaining using monoclonal antibody 34βE12 (37). As judged by 2D-PAGE Western immunoblotting this antibody also exhibits a wider cross-reactivity with CKs 14 and 19 (results not shown).

Type 4 Heterogeneity

This type of heterogeneity was observed in only one UP that showed extensive areas with CK7 negative cells, a result that

was confirmed by 2D gel analysis of protein extracts prepared from the same tumor. The lesion did not express CK14 and showed normal staining and expression of CK8 suggesting that its origin is most likely not connected to squamous differentiation.

Type 5 Heterogeneity

Even though aberrant expression of CK20 has been reported earlier in papillary tumors (9), the absence of staining of the basal cell layer with CK20 antibodies has not been reported. The characteristic future of type 5 heterogeneity is the absence of CK20 staining in the basal cell layer. In addition, one of the lesions revealing this type of heterogeneity expressed CK14 in the basal layer that was confirmed by 2D gel analysis. The biological potential of this type of heterogeneity is at present unclear, although the expression of CK14 may reveal the early events in squamous differentiation. The origin of the type 5 heterogeneity is at present unclear, although the one expressing CK14 may be undergoing early events in squamous differentiation.

Currently, we are carrying out a systematic analysis of low grade non-invasive and invasive TCCs with the panel of antibodies in an effort to determine the incidence of the various lesions described above at various stages of cancer development. In addition, we are contemplating the idea of using laser capture microdissection to obtain distinct populations of cells exhibiting particular types of heterogeneities for proteomic and microarrays analysis (15, 32, 33). The aim of these studies is to identify additional markers that may prove instrumental for forthcoming studies.

Presently, it is not possible to comment on the correlation, if any, between the presence of a given type of heterogeneity and the rate of recurrence or progression to invasive disease as the number of patients was relatively small, and the follow-up was too short. Twenty-nine percent of the patients experienced recurrences during the course of the study, a number that is very close to those reported in previous studies (8). Interestingly, patients harboring phenotypic alterations in the basal cell compartment (types 3 and 5) showed the highest number of recurrences (4/7 and 2/3, respectively), and all type 3 lesions progressed to a higher degree of dedifferentiation. Evaluation of the biological potential of these and other lesions characterized in this study will require a much larger sample size, as well as a follow-up of up to 10 years. In addition, these studies must take into consideration the biological significance of having combinations of various types of tumor heterogeneities as invasive lesions are known to exhibit areas with different histological grades (38).

Finally, we would like to stress that the approach presented here for revealing tumor heterogeneity has already been used successfully to identify metaplastic and premalignant lesions in bladder squamous cell carcinomas using immunowalking (18). Its full potential, however, would only be realized when a comprehensive set of antibodies against marker proteins is used in combination with tissue arrays (39) in a high throughput fashion.

* This work was supported in part by grants from the Danish Cancer Society and the Danish Biotechnology Program and by fellowships from the Danish Cancer Society (to M. Ø. and H. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Deceased July 7, 1998.

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