Loss of Adipocyte-type Fatty Acid Binding Protein and Other Protein Biomarkers Is Associated with Progression of Human Bladder Transitional Cell Carcinomas

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ABSTRACT

Multifocal recurrent papillary tumors provide a unique model system to study the molecular mechanisms underlying the steps involved in transitional cell carcinoma progression and offer a valuable source of material to search for biomarkers that may form the basis for diagnosis, prognosis, and treatment. We have examined the protein expression profiles of normal bladder urothelium and of 63 transitional cell carcinomas of various histopathological grades and T stages using high-resolution, two-dimensional gel electrophoresis, microsequencing, mass spectrometry, and a two-dimensional gel protein database approach for polypeptide identification (http://biobase.dk/cgi-bin/celis). In general, the results revealed a striking similarity between the overall qualitative expression patterns of papillary tumors of all grades, as well as of papillary and solid tumors of grade III. With few exceptions, tumors of grades I–III expressed, albeit at different levels, all of the keratins (7, 8, 13, 17, 18, 19, and 20) found in the normal urothelium. Grade IV tumors lacked or expressed reduced levels of keratin 13 but most resembled low-grade tumors. One invasive grade IV tumor, however, expressed a fibroblast-like protein phenotype. Four proteins that were expressed by normal urothelium and were lost at various stages of progression were identified as glutathione S-transferase μ, prostatic acid phosphatase (ACP), and keratin 13. The percentage of tumors expressing A-FABP was very high in low-grade lesions but decreased drastically (P = 0.0006) in grade III and IV neoplasms. In addition, low-grade tumors contained more A-FABP than their high-grade counterparts. The stage of the disease was also statistically (P = 0.0269) related to the presence or absence of A-FABP in grade III tumors. Similar analysis of glutathione S-transferase μ and PGDH showed a statistically significant decrease of these proteins in high-grade (grades III and IV) tumors (P = 0.0026 and P = 0.0044, respectively). Only PGDH showed a suggestive correlation (P = 0.0775) with the stage of the disease in grade III tumors. Keratin 13 showed a drastic decrease in grade IV tumors. In addition to identifying biomarkers that may have prognostic value, our studies have suggested that A-FABP is an important component of the pathway(s) leading to bladder cancer development.

INTRODUCTION

Bladder cancer comprises a broad spectrum of tumors with various histological types, including TCCs, adenocarcinomas, and SCCs (1, 2). TCCs are by far the more prevalent tumors and represent nearly 95% of all bladder cancers in the Western hemisphere (3). About 70% of the urinary bladder TCCs are diagnosed at presentation as well-differentiated superficial lesions (papillary tumors) that are confined either to the mucosa (grade T1) or to the underlying connective tissue (grade Ta). The rest correspond to highly invasive (stages T2–4), poorly differentiated tumors. In many cases, dysplasia and/or CIS are detected in the surrounding mucosa in addition to the main tumor.

Superficial bladder cancer represents a heterogeneous group of tumors, about 60% of which will have one or more recurrences, both in time and space, after transurethral resection (4). Of these, some will progress to invasive and/or metastatic disease and are therefore potentially lethal. This scenario is in harmony with the notion that bladder cancer is a “field disease” (5), that is, the whole bladder urothelium that is in contact with the carcinogen is at risk of developing cancer, although any given lesion is clonal in its origin. Thus, multifocal recurrent papillary tumors provide a unique model system to study the molecular mechanisms underlying the various steps involved in cancer development, and offer a valuable source of material to search for specific markers for early tumor detection (1, 6).

Considering the recent progress in tumor biology and the well-known correlation between neoplastic progression and genetic alterations (7, 8), it is not surprising that most research in the field of bladder cancer has been focused on the identification of oncogenes and tumor suppressor genes. Oncogenes that have been associated with bladder cancer include Ha-ras, erbB1 and erbB2, c-myc, and src (8, 9). Ten % of bladder cancers have been shown to have a ras gene in a mutated form (8), and it has been suggested that p21 expression may serve as a prognostic factor for the malignant potential of a bladder lesion (8). Cytogenetic studies, on the other hand, have revealed multiple chromosome abnormalities in bladder cancer. To date, several novel suppressor loci on chromosomes 3p, 4p, 4q, 5q, 8p, 9p, 9q, 11p, 13q, 14q, 17p, and 18q (8, 9) have been identified, and a few have been located at a genetic distance that permits positional cloning. As a whole, these studies have not only supported the notion that bladder cancer is a multistep process but also have provided compelling evidence for a role of p53 in TCCs (8, 13, 14).

In general, bladder cancer research at the protein level has lagged behind genetic studies, mainly because of the complexity of the technology required to separate, analyze, and identify the thousands of polypeptides present in the tumor tissue. Proteins frequently are the functional molecules and, therefore, are most likely to reflect qualitative (expression of new proteins or change in posttranslational modifications) and quantitative (down-regulation or up-regulation) differences associated with different stages of cancer development. Most studies thus far have been focused on the search for factors, often identified in other systems, that may have prognostic value. To name a few, these include keratins, blood group antigens (ABH and T), cell proliferation markers (proliferating cell nuclear antigen and Ki 67), tumor suppressors (p53 and RB proteins), oncogene products (ras and c-erb-2), and differentiation-related proteins (uroplakin III, EGF-receptor, and G-actin; Refs. 6, 8, 15–18, and references therein). Without exception, however, there is no single prognostic factor that is able to predict recurrence and progression in an individual patient (6).
LOSS OF A-FABP CORRELATES WITH PROGRESSION OF BLADDER TCCs

In our laboratories, we have considered the possibility of using protein expression profiles of tumors (as a whole or in part) as fingerprints to subclassify histopathological types and as a starting point toward a systematic search for protein markers that may form the basis for diagnosis, prognosis, and treatment. The feasibility of this approach has been tested recently in a limited study of bladder urothelial SCCs (19), as cells in these tumors resemble keratinocytes, a cell type for which we have identified 35% of the 3154 proteins detected by high-resolution 2D gel electrophoresis (20; see also Internet URL http://biobase.dk/cgi-bin/celis). Using the latter technique in combination with data stored in comprehensive 2D gel databases of keratinocyte (20) and urine proteins (21), it has been possible to identify a single biomarker, psoriasin (22), which is expressed specifically by SCCs and externalized to the urine of these patients (19).

In parallel to the SCC studies, we carried out a similar analysis of the protein expression profiles of TCCs of various gradings and stages with the long-term goal of developing a more accurate classification of superficial lesions that may facilitate the identification of individuals at risk and, in addition, provide a model system for studying the molecular mechanisms underlying progression from noninvasive to invasive and metastatic lesions. Thus far, nearly 200 tumors have been analyzed by 2D gel electrophoresis, and many have been highly informative. Currently, a 2D gel database of TCC proteins is being constructed that lists hundreds of known proteins and that aims at integrating protein (identity, cellular localization, function, levels under various physiological conditions, coregulated proteins, partial protein sequences, and others) and DNA mapping and sequencing information (http://biobase.dk/cgi-bin/celis). Here, we report the first outcome of the TCC studies, which have led to the identification of abundant protein biomarkers with patterns of expression that correlate with tumor progression.

MATERIALS AND METHODS

Tumor Biopsies and Processing. TCCs removed at the Department of Urology during 1994 and 1995 were collected (if informed consent was obtained from the patients) and examined in this study, which was approved by the local ethical committee. Upon receipt, tumors were dissected carefully with the aid of a scalpel and homogenized (glass homogenizer) in a small volume of lysis solution (23). Samples were kept at −20°C until used.

Labeling of Tumors and Normal Bladder Urothelium with [35S]Methionine. Microscopically pure TCCs were minced in small pieces with the aid of a scalpel and labeled for 14–16 h in a 10-ml sterile plastic conical tube containing 0.2 ml of modified Eagle's medium lacking methionine and containing 2% dialyzed (against 0.95% NaCl) FCS and 50 μCi of [35S]methionine (SJ204, Amersham; Ref. 24). At the end of the labeling period, the pieces were centrifuged at 2000 × g for 2 min, resuspended in 0.3–0.4 ml of lysis solution, and homogenized in a small glass homogenizer. Samples were stored at −20°C until used. Normal bladder urothelium was scraped with the aid of a scalpel and labeled with [35S]methionine as described above.

Antibodies. Rabbit antisera were raised against Coomassie Brilliant Blue-stained A-FABP, GST-μ, and keratin 13 recovered from fixed, dried 2D gels of tumor 532-1 according to standard procedures. The antibodies against keratin 7 and 18 were a gift from F. Ramaekers (Nijmegen, the Netherlands). The vimentin antibody was purchased from DAKO.

Statistical Methods. The χ² test with Yates correction for small numbers was used for comparison of patient groups.

Other Procedures. The procedures for 2D gel electrophoresis (24), immunoblotting and enhanced chemiluminescence detection (25), immunofluorescence (19), microsequencing (26), mass spectrometry (27), and database searching have been described in detail elsewhere.

Fig. 1. IEF 2D gel of [35S]methionine-labeled proteins from normal bladder urothelium. Protein biomarkers associated with tumor progression are indicated in red. αtubulin; βtubulin; K, keratin; V, vimentin.
Table 1 Microsequencing of biomarker proteins

<table>
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<tr>
<th>Protein name</th>
<th>Database entry</th>
<th>M_r</th>
<th>pI</th>
<th>Amino acid sequence</th>
<th>Amino acid residues verified by mass spectrometry</th>
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<tr>
<td>PGDH</td>
<td>PGDH_HUMAN</td>
<td>28,977</td>
<td>5.7</td>
<td>52-LGLDPNLYLIDGAHK-68</td>
<td>7-19, 20-28, 47-57, 58-72, 73-76, 77-84, 156-163, 250-264</td>
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- *Swissprot database.
- a Calculated from the database entry using the Peptidesort program of the GCG Wisconsin package (Version 8, September 1994, Genetics Computer Group).
- b Single-letter code.
- c Matrix Assisted Laser Desorption Ionization analysis of purified tryptic peptides or total digest.

RESULTS

Protein Expression Phenotype of Normal Bladder Urothelium.
A high priority of our studies was to characterize the protein expression profile of the normal bladder urothelium. To minimize treatment, we opted for scraping the urothelium from the bladder wall with the aid of a scalpel rather than detaching it by using enzyme digestion. The procedure, which yielded only small amounts of urothelium, was simple but required overnight labeling of the tissue with [35S]methionine prior to 2D gel electrophoresis. As shown in the representative IEF 2D gel autoradiogram presented in Fig. 1, the transitional epithelium expresses both type I (keratins 7 and 8) and type II keratins (keratins 13, 17, 18, 19, and 20; Ref. 15) but lacks vimentin [the position to which vimentin (V) migrates is indicated as reference]. The identity of these proteins was verified by microsequencing (only the data for keratin 13 are given in Table 1) and their presence (or absence, in the case of vimentin) in the bladder urothelium was further confirmed by indirect immunofluorescence staining of formaldehyde-fixed cryostat sections. Fig. 2 shows photomicrographs of sections reacted with antibodies against keratin 7 (Fig. 2A, even staining of all layers), keratin 13 (Fig. 2B, weaker staining of umbrella cells), keratin 18 (Fig. 2C, mainly umbrella cell staining), and vimentin (Fig. 2D, no staining of the urothelium).

For orientation and to facilitate the discussion, we have indicated, in Fig. 1, the positions of a few proteins, including GST-µ, PGDH, A-FABP, and keratin 13, which have been identified by microsequencing (Table 1), 2D gel immunoblotting, and matching to the master gel in the human keratinocyte 2D gel protein database (20; see also Internet URL http://biobase.dk/cgi-bin/celis).

Protein Phenotype of TCCs of Various Histopathological Grades and T Stages; Identification of High-Abundance Biomarkers. One hundred sixty-three suspected TCCs removed at the Department of Urology, Skejby Hospital, between 1994 and 1995, were

Fig. 2. Indirect immunofluorescence staining of formaldehyde-fixed cryostat sections reacted with antibodies against various intermediate filament proteins. A—D, normal bladder urothelium; E—H, TCC 532-1, grade II, Ta; I—L, TCC 514-1, grade IV, T2-4; M—P, TCC 520-1, grade IV, T2-4.
analyzed by high-resolution 2D gel electrophoresis and Coomassie Brilliant Blue staining. In a few cases, small tumor pieces were labeled overnight with [35S]methionine prior to gel electrophoresis. In all, 63 TCCs of various histopathological grades and T stages (5 grade I, Ta; 25 grade II, Ta; 1 grade II, T2-4; 2 grade III, Ta; 12 grade III, T1; 13 grade III, T2-4; 5 grade IV, T2-4) yielded acceptable protein profiles both in terms of their purity as assessed by monitoring for the absence of vimentin (contamination with connective tissue) and desmin (contamination with smooth muscle cells) and polypeptide resolution.

Figs. 3 and 4 show autoradiograms of [35S]methionine-labeled proteins synthesized by tumors displaying representative patterns. To provide an estimate of the level of abundance of the different proteins detected in the autoradiograms, we have included in Fig. 5 Coomassie Brilliant Blue-stained gels of TCCs 519-1 (Fig. 5A, grade I, Ta) and 521-1 (Fig. 5B, grade I, Ta; compare with autoradiograms in Fig. 3, A and B).

As shown in Fig. 3, the overall protein expression profiles of grade I, II, and III (Ta and T1) tumors are very similar and, with some exceptions, most of them expressed, albeit at different levels, all of the keratins found in the normal urothelium (compare with Fig. 1). Most of the tumors lacked vimentin, as confirmed by indirect immunofluorescence (Fig. 2H, 532-1), and displayed substantial levels of stratifin (28), which underlay their epithelial origin. A large fraction of the grade I and II lesions exhibited a high content of keratins, particularly keratins 13 and 19, which individually exceeded the levels of total actin (compare Figs. 5A, 519-1, and 5B, 521-1). The difference in keratin content, however, was not obvious from the immunofluorescence staining of sections reacted with keratin antibodies (results not shown).

Grade IV tumors (as well as some invasive grade III lesions), on the other hand, were more heterogeneous in their profiles, as depicted in Fig. 4, A and B. The protein expression patterns of most of these neoplasms were similar to those of grade I, II, and III lesions (Fig. 4A, B).
514-1), whereas one exhibited a strikingly different protein fingerprint that resembled very much that of fibroblasts (29) and that most likely reflects epithelial-mesenchymal transition (Fig. 4A, 520-1, grade IV, T2-4), a phenomenon that is often observed late in progression of human carcinomas (30). Cells in this tumor synthesized vimentin and expressed low levels of keratins (as determined by double immuno-

![Fig. 4. IEF 2D gels of \[^{35}S\]methionine-labeled proteins from grade IV TCCs. A, TCC 514-1, grade IV, T2-4; B, TCC 520-1, grade IV, T2-4.](image)

![Fig. 5. IEF 2D gels of TCC proteins visualized by Coomassie Brilliant Blue (A–F) or silver staining (G and H). A, TCC 519-1, grade I, Ta; B, TCC 521-1, grade I, Ta; C–F, examples of gels scored as negative (C), weak (D), ± (E), and + (F) for A-FABP. G, TCC 527-1, grade III, T1; H, TCC 527-II, grade III, T2-4. Both TCCs 527-1 and II were resected from the same individual at the time of presentation.](image)
fluorescence, not shown) and the epithelial marker stratifin (Fig. 4B).
In addition, they expressed the neuron-specific protein PGP 9.5, a polypeptide that we have identified previously in normal human MRC-5 fetal lung fibroblasts (29). Protein gene product (PGP) 9.5 was identified by immunoblotting (results not shown) and by comparison with the master gel in the MRC-5 fibroblast protein database (29). Fig. 2, I—L and M—P, shows immunofluorescence pictures of the two types of grade IV tumors reacted with keratins 7, 13, and 18, as well as with vimentin antibodies. For comparison, Fig. 2, E—H, shows similar stainings of a grade II tumor (532-1).

Careful comparison of the protein profiles of the various tumors with those of the normal urothelium revealed a few proteins, including keratin 13, that were lost at different stages of tumor development. Of these, three abundant polypeptides that were overexpressed in some low-grade neoplasms were selected for further analysis as their levels could be assessed in nearly all of the 63 tumors analyzed. The three putative markers were cut from Coomassie-stained gels and microsequenced to assess their identity (Table 1; upper three proteins). Comparison of the peptide sequences, and in some cases of their masses, with sequences stored in protein and nucleic acid databases indicated that these proteins share identical peptides with GST-@ (31), 15 hydroxy PGDH (32), and A-FABP (33). Although the homologies are 100% for all three proteins, we cannot exclude the possibility that they may correspond to new variants, because only 2–3 tryptic peptides were analyzed in each case (Table 1). In the case of FABP, however, comigration and cloning experiments confirmed that this protein corresponds to A-FABP.

The normal bladder urothelium, as well as most of the tumors analyzed by 2D gel electrophoresis, expressed PA-FABP in addition to A-FABP (Fig. 1). Immunoblotting experiments using rabbit polyclonal antibodies raised against pure A-FABP recovered from fixed, dried Coomassie Brilliant Blue-stained gels showed that this protein shares epitope(s) with PA-FABP (Fig. 6B). Similar antibodies raised against GST-@ did not react with GST-@ (Fig. 6D), the predominant isoform in normal bladder urothelium. Interestingly, a few of the tumors expressed a polymorphic form of GST-@ (GST-@), which exhibits a similar apparent molecular weight but has a slightly more acidic isoelectric point (pI) (Fig. 6F, compare with Fig. 6E). The identity of this protein was confirmed by microsequencing (results not shown) and 2D gel immunoblotting using rabbit GST-@ antibodies (results not shown).

Other down-regulated proteins of interest included MnSOD (Fig. 1), as well as two low-abundance proteins of yet unknown identity (Fig. 1, arrows). These proteins are down-regulated in all of the grade I tumors and may represent early markers of transformation. Their proper analysis, however, must await further studies, including larger numbers of grade I tumors.

**Biomarker Distribution in Low-Grade and High-Grade Tumors.** Table 2 shows the levels of expression of the four markers (A-FABP, GST-@, PGDH, and keratin 13) in the 63 tumors analyzed. The data were scored entirely based on the visual analysis of Coomassie Brilliant Blue-stained gels. In the case of A-FABP, tumors scored as positive differed significantly with respect to the levels of this protein. For the record, Fig. 5, C—F, shows Coomassie Brilliant Blue-stained gels that were scored as either negative (Fig. 5C) or positive (weak to +, Fig. 5, D—F) for this protein. The levels of A-FABP in the normal urothelium ranged from weak to ± (no Coomassie-stained gel is shown, but see the autoradiogram in Fig. 1).

As stated above, some of the low-grade tumors (I and II) overexpressed A-FABP (see, for example, Figs. 3A and 5A). With few exceptions, the overexpression levels of PGDH were very similar in all cases studied. Likewise, the induction of GST-@ observed in low-grade TCCs resulted in similar protein expression levels.

As shown in Table 2, the percentage of tumors expressing A-FABP was high in low-grade lesions but decreased significantly (P = 0.0006; grade I and II versus III and IV) in grade III and IV neoplasms. In addition, low-grade tumors contained more A-FABP than their high-grade counterparts (not shown). The stage of the disease was also significantly (P = 0.0269; stage Ta and T1 versus T2-4) related to the presence or absence of A-FABP in grade III tumors (Table 2). To illustrate this point, Fig. 5G (527-1, grade III, T1) and Fig. 5H (527-11, grade III, T2-4) show silver-stained 2D gels of two grade III tumors differing only in their T stage (T1 and T2-4).

These tumors, which were resected simultaneously from the same individual at first presentation, showed a dramatic difference in their content of A-FABP. Apart from A-FABP, the overall protein expres-
tion patterns of these tumors were strikingly similar (compare Fig. 5, G and H), despite the fact that one was papillary (527-1) and the other was solid.

Similar analysis of GST-μ and PGDH showed a statistically significant decrease of these proteins in high-grade tumors (P = 0.0025 and P = 0.0044, respectively; grades I and II versus grades III and IV) as depicted in Table 2. Of these proteins, only PGDH showed a suggestive correlation (P = 0.0775; stage Ta and T1 versus T2-4) with the stage of the disease in grade III tumors (Table 2). The results regarding keratin 13 were straightforward, as the levels of this protein decreased dramatically in grade IV tumors (Table 2). Although it was not possible to detect this protein by Coomassie Brilliant Blue staining, the immunofluorescence studies showed above-background levels of staining as well as the presence of isolated groups of positive cells (Fig. 2, J and N). These apparently discrepant observations reflect differences in sensitivity of the two techniques as applied in this study.

**DISCUSSION**

TCCs are subdivided into noninvasive papillary and nonpapillary invasive carcinoma types (1, 2) that are believed to originate from different genetic alterations (4, 8, 14). Papillary TCCs correspond to 70% of all TCCs and usually are of low grade and are noninvasive at the time of presentation (3). These tumors begin as areas of hyperplasia that later undergo a process of dedifferentiation (grades I-IV). Invasive tumors may arise from these lesions, although poorly differentiated neoplasms (grade III and IV) have a higher tendency to invade and metastasize (2). Nonpapillary invasive carcinomas, on the other hand, are believed to develop from CIS, a flat lesion of uncertain biological behavior that can be associated with high-grade invasive tumors (34). CIS neoplasms are usually of high grade (III) and are detected only late in the disease, often with invasion. Added to the complexity of TCCs is the fact that a preponderance of high-grade tumors (infiltrating or recurrent carcinomas) have a tendency to undergo metaplasia (squamous and/or glandular; Refs. 1 and 2 and references therein) as well as epithelial-mesenchymal transitions (30). The latter may occur in defined areas as well as in substantial parts of the tumor and, in addition, may vary from area to area with respect to their degrees of differentiation. Cytogenetic studies and molecular genetic data have indicated that chromosomes 9 and 17 are frequently altered in bladder urothelial tumors (8, 11, 12, and references therein), and there is compelling evidence suggesting that the tumor suppressor p53 (8, 13, 14) is involved in the development and progression of TCCs. Recently, Spruck et al. (14) showed that chromosome 9 deletions occur early during progression of papillary tumors leading to a "hyperplastic stage," whereas p53 mutations appear later in the process and confer invasive properties. The situation, however, is reverse in the case of CIS, as a large fraction of these lesions contain p53 mutations. Chromosome 9 alterations in these tumors have been shown to occur later or not at all (14, 35). In addition to pointing toward two divergent pathways of bladder tumor progression, these studies implied that the order in which the genetic changes take place is important in determining the outcome of the lesion. Clearly, a complete understanding of TCCs will require knowledge of the molecular mechanism underlying both the genetic and the epigenetic factors affecting neoplasia in the urothelium, as well as of the properties of the wide spectrum of different tumors arising from this tissue (SCCs, adenocarcinomas, and others). The heterogeneity of TCCs poses a great problem in therapy and, therefore, it is of the utmost importance to derive probes that may identify transitional epithelial cells at various stages of differentiation and invasion as well as cells of different histological types.

In this study, we have presented our initial attempt to fingerprint and partially characterize the protein expression profiles of normal bladder urothelium and TCCs of various grades and T stages with the long-term aim of revealing markers that may form the basis for diagnosis, prognosis, and treatment. With few exceptions, the results revealed a striking overall similarity between the qualitative expression patterns of papillary tumors of all grades, as well as of papillary and solid tumors of the same grade (see Fig. 5, G and H). One invasive grade IV tumor, however (520-1, sarcomatoid), exhibited a clear fibroblast-like protein pattern that most likely reflected epithelial-mesenchymal transitions often observed late in progression of carcinomas (30). The tumor expressed low levels of keratins and the epithelial marker stratatin (Fig. 4H) but exhibited vimentin as well as the neuronal protein PGP 9.5, a polypeptide that we have described previously in normal human MRC-5 embryonal lung fibroblasts (29). PGP 9.5 is highly down-regulated in SV40-transformed human fibroblasts (29), indicating that cultured cells may not necessarily represent an appropriated model to study the biochemical changes underlying invasiveness. Expression of a neuronal marker (neuron-specific enolase) by advanced TCCs (36) as well as by small cell carcinomas of the urinary bladder (37) has been reported earlier.

Of the four abundant biomarkers (A-FABP, GST-μ, PGDH, and keratin 13) identified in this study, A-FABP is perhaps one of the most interesting, as its presence correlated both with the grade of atypia (P = 0.0006) and the stage of the disease (P = 0.0269). This low molecular weight protein is a member of the cytosolic multigene family of lipid-binding proteins that includes heart, liver, intestinal, muscle, brain, and epithelial isoforms (38 and references therein). The precise functions of members of the FABP family are unknown, although there is evidence suggesting that they may play roles in intracellular lipid transport and metabolism, as well as in signal transduction (39, 40, and references therein). The latter has been inferred from the fact that long-chain fatty acids and their metabolites can act as primary and secondary messengers in specific signaling pathways (41). Moreover, A-FABP is partially phosphorylated on Tyr19 (42), and there are data suggesting that the phosphorylated variant may participate in the insulin-signaling cascade (43). Interestingly, members of the FABP family are expressed highly in differentiated cells, and there is compelling evidence showing that both the heart isoform and the MDGI, which is also a member of this family (44), inhibit growth of cultured normal mouse mammary cells (45). In
organ cultures, MDGI induces differentiation and stimulates milk protein production. In addition, MDGI provokes its own expression and inhibits the mitogenic effect of epidermal growth factor, a fact that has strengthened its putative role as a differentiation factor in the mammary gland (45). Recently, Huynh et al. (46) have shown that MDGI functions as a potent tumor suppressor in transfected human breast cancer cells and suggested that this protein is a putative candidate for the Ip breast tumor suppressor gene. Our own studies of human keratinocytes have shown that the levels of PA-FABP increase dramatically in cultured normal epidermal cells induced to differentiate via an abnormal pathway (47).

Given the growth inhibitory properties displayed by some members of the FABP family, it was not surprising to find that some noninvasive grade I and II TCCs expressed high levels of A-FABP, as cells in these lesions have a limited proliferative capacity and are most likely defective in step(s) that are essential for orchestrating the delicate balance between proliferation and differentiation. Thus, the overexpression A-FABP (and maybe that of PGDH and GST-μ; see below) observed in several low-grade tumors may reflect an attempt by these cells to curtail growth. This situation is in some ways similar to that observed in hyperproliferative psoriatic keratinocytes, which proliferate in a controlled fashion and overexpress some differentiation markers (including PA-FABP) that are present at low levels in normally differentiated epidermal cells (22, 47). Grade II TCCs, which occasionally progress toward a more undifferentiated phenotype, however, may down-regulate or cease to synthesize A-FABP because only 37% of the grade III and none of the grade IV tumors expressed this protein (Table 2). In addition, only 15.8% of all of the invasive tumors analyzed expressed this protein. Moreover, all of the established bladder cell lines analyzed thus far by 2D gel electrophoresis (T24, Hu 609, and HCV29) have failed to show significant levels of this protein, confirming that its expression may not be compatible with the immortal phenotype (48; results not shown). Taken as a whole, the above observations imply that loss of A-FABP may have a prognostic value and may argue for a putative role for this protein as a primary or secondary growth inhibitor that may be required for normal urothelial differentiation. Experiments are currently under way with the aim of producing a knockout mouse model to study its role in bladder urothelial differentiation.

The striking induction of GST-μ observed in some low-grade tumors is noteworthy, as this protein belongs to a multigene family of detoxification enzymes that catalyze the conjugation of glutathione to a large number of compounds having an electrophilic center and that include alkylating agents as well as other types of drugs used in cancer therapy (49 and references therein). GST enzymes have been involved in anticancer drug resistance in general, although the induction of GST-μ has been associated directly with nitrosoureas (49). Most, if not all, studies, however, have been carried out primarily with transfected cultured mammalian cells, and often these studies have yielded contradictory results. Whether GSTs have a direct or indirect role in drug resistance remains to be elucidated; however, our studies have underlined the need to address this possibility when contemplating chemotherapy in bladder TCCs, as the levels of this protein may predict chemotherapeutic responsiveness.

Our studies also revealed an important increase in the amounts of PGDH in some low-grade tumors. The levels of PGDH correlated both with the grade of atypia (P = 0.0044) and stage of the disease (P = 0.0775). PGDH is the main enzyme involved in the catabolic pathway of prostaglandins and catalyzes the initial reaction in the conversion of biologically active prostaglandins to their inactive analogues, 15-keto-metabolites (50). The enzyme is very abundant in placenta, a tissue that has a large capacity for inactivating prostanoids (51), but it has not been reported in the bladder urothelium. Furthermore, changes in the levels of prostaglandin E2 have been associated with promotion of rat bladder carcinogenesis (52), a fact that has indirectly involved PGDH in this process.

The drastic decrease in the levels of keratin 13 observed in grade IV tumors is not novel, as others (15, 53) have reported previously that highly invasive TCCs show greatly reduced levels of this protein and stain focally when reacted with specific antibodies. The loss of this keratin in high-grade neoplasms may vary from tumor to tumor or even within a tumor due to metaplasia, but it is expected to be more severe in highly anaplastic lesions undergoing extensive epithelial-mesenchymal transition (30). The highest degree of keratin loss has been noticed in areas of muscle invasion, and therefore, antibodies against this protein should be valuable in assessing tumor progression in TCCs.

As mentioned earlier, one of the problems associated with the study of bladder TCCs is the presence of squamous and/or glandular metaplasia in high-grade tumors. Although this has not been a problem in this particular study, we have carried out parallel analysis of the protein profiles of SCCs (19) and adenocarcinomas with the aim of identifying specific biomarkers of squamous and glandular metaplasia that may facilitate the histopathological grading and prognosis of TCCs in general. Thus far, the SCC studies have led to the identification of psoriasin, a low molecular weight calcium-binding protein (22) of restricted tissue distribution that is expressed specifically by SCCs (19). Psoriasin is externalized to the urine of these patients and has provided a putative recurrence marker during follow-up (19). Adenocarcinomas of the bladder are rare; therefore, their analysis is still at a very early stage.

In the long run, a practical goal of our studies is to identify a complete set of protein biomarkers that may be useful to classify histopathological grades of TCCs and that will provide specific probes (antibodies for immunohistochemical analysis of paraffin-embedded tissue samples and for urine-based ELISAs) for the objective diagnosis, prognosis, and treatment of these lesions. These putative biomarkers will also serve as landmarks for forthcoming research aimed at further dissecting the various stages involved in tumor progression. Another long-term priority of our research includes a search for genes involved in invasiveness and metastasis. The direct analysis of protein profiles may not be sensitive enough to reveal components with very low abundance; therefore, this problem is being approached by chemical cross-linking subtraction (54) using mRNA extracted from tumors, resected from the same patient at the time of presentation, that differ only in their T stages.

Finally, we would like to stress that our studies have thus far mainly focused on papillary TCCs, as these lesions provide enough material for both gel and protein sequence analysis. Improvements in the [35S]methionine labeling technology, however, has now made possible a systematic analysis of CIS as well as of urothelium biopsies exhibiting various grades of atypia. These studies, which are currently under way, may eventually reveal markers that reflect the early emergence of the malignant phenotype.

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