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Persistent Uroplakin Expression in Advanced Urothelial Carcinomas: Implications in Urothelial Tumor Progression and Clinical Outcome

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Abstract

As the terminal differentiation products of human urothelium, uroplakins (UPs) would be expected to diminish during urothelial tumorigenesis. Surprisingly, recent studies found UPs to be retained even by well-advanced urothelial carcinomas, suggesting that the loss of UPs does not strictly parallel urothelial transformation. Little is known, however, about whether the status of UPs is associated with a particular pathological parameter, tumor's biological behavior or patient outcome. Here we assessed UP expression by immunohistochemistry on tissue arrays from 285 patients with bladder urothelial carcinomas or non-tumor conditions. UPs were expressed in all 9 normal urothelial specimens, 63/74 (85%) patients with non-muscle-invasive urothelial carcinomas on transurethral resection, 104/202 (51.5%) patients who underwent radical cystectomy for advanced urothelial carcinomas, and 33/50 (66%) lymph node metastases. Normally associated with urothelial apical surface, UPs were localized aberrantly in tumors, including micro-luminal, basal-laminal, cytoplasmic or uniform patterns. In non-muscle-invasive diseases, there was no association between UP expression and disease recurrence, progression or mortality. In contrast, in invasive diseases, absent UP expression was significantly associated with advanced pathologic stage, lymph node metastases, disease recurrence and bladder cancer-specific mortality ($p=0.042$, $p=0.035$, $p=0.023$ and $p=0.022$, respectively) in univariate analyses. Furthermore, UP status was independent of key cell-cycle regulators, including p53, pRb, p27 and cyclin D1, thus excluding a functional link between these two groups of proteins. Our data demonstrate for the first time that persistent UP expression is

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associated with a favorable clinical outcome and that UPs may be used as adjunct markers for predicting the prognoses of patients with invasive and metastatic bladder carcinomas. Our results also suggest that UP-positive and -negative carcinomas have different clonal origins or may be derived from different cancer stem cells.

Keywords

bladder cancer; uroplakin; progression; prognosis; transitional cell carcinoma

INTRODUCTION

Urothelial carcinomas of the bladder are among the most common human malignancies, ranked fourth in incidence in all male cancers and ninth in all female cancers in the United States. According to the American Cancer Society, over 61,000 new cases were diagnosed in 2006, of which 13,000 will die of this disease (1). Globally, approximately 336,000 new cases are diagnosed annually, resulting in 132,000 deaths (2). Fortunately, not all urothelial carcinomas carry the same risk of reaching the incurable stage. In fact, there is increasing evidence to suggest that at least two major carcinoma variants exist: the low-grade, superficial papillary tumors and the high-grade invasive tumors (3–9). The former frequently recur, but relatively few of them progress to muscle-invasive stages. In contrast, the latter are derived mainly from flat carcinoma in situ and, less often, from high-grade papillary tumors. They follow an aggressive course, with at least half of the cases eventually progressing with local and distant metastases (10). This occurs in an unpredictable manner and despite the complete removal of the primary carcinomas by radical cystectomy, thus presenting a major challenge in curing this disease (11). In this regard, it is critically important to be able to predict the biological behavior and the likelihood of progression of a given carcinoma at presentation, as this will greatly facilitate an appropriate treatment decision. However, the number and type of prognostic markers available for this purpose remain insufficient.

Uroplakins (UPs) are a group of integral membrane proteins that are synthesized during an advanced stage of human urothelial differentiation (12). Thus far, four major UPs (UPIa, Ib, II and IIIa) and one minor UP (UPIIb) have been identified (12,13). Together, these proteins constitute the major protein building blocks of the urothelial plaques, also named asymmetric unit membranes (or AUMs) that cover over 90% of the urothelial surface (14,15). Topologically, UPIa and Ib span the plasma membrane four times and belong to a growing family of structurally conserved but functionally elusive proteins all possessing four transmembrane domains (tetraspanins). UPII and IIIa, however, contain only one transmembrane domain, which divides a large luminal domain and a smaller cytoplasmic tail. As demonstrated by chemical crosslinking and in vitro transfection analyses, the four major UPs form two distinct pairs (UPIa with II and UPIb with IIIa), in order for them to exit efficiently from the endoplasmic reticulum during biosynthesis (16,17). Because UPs are highly conserved during mammalian evolution (12), it has been suggested that they play key roles in urothelial functions, including participation in the permeability barrier, adjustment of urothelial surface area, stabilization of the urothelial surface and development of the urinary tract (12,18,19). Consistent with their proposed functions at the urothelial surface, UPs are expressed in a tightly differentiation-dependent manner, being detected ultrastructurally (in the form of AUMs) and immunohistochemically at the apical surface and cytoplasm of the superficial cell layer of human urothelium with little or no detection in the intermediate and basal layers (20,21).

The fact that UPs are the major differentiation products of urothelium strongly implies that UPs would be significantly down-regulated during urothelial transformation and

tumorigenesis. This was proven to be the case in cultured normal urothelial cells, where incomplete differentiation and/or hyper-proliferation led to a profound reduction of all UPs, particularly on a protein level (22). Similarly, in a number of human bladder cancer cell lines, UPs are either down-regulated (RT4, RT112) or undetectable (J82, T24) (23) [unpublished observation]. These results suggest that, like many epithelial differentiation markers, UP expression is profoundly perturbed and/or down-regulated during urothelial transformation. Surprisingly, however, this idea is not supported by data from human urothelial carcinomas. For example, Moll et al showed by immunohistochemical staining that over half of the invasive and metastatic urothelial carcinomas continued to express UPs (20). Their results, combined with those of the subsequent studies from several independent groups (21,24), strongly suggest that UP down-regulation does not strictly parallel urothelial tumorigenesis and progression. Most of the existing studies, however, assessed a relatively small number of urothelial carcinomas, with no correlation with clinical outcome. Key questions remain as to whether the status of UP is associated with a particular pathologic parameter, carcinoma's biologic behavior or clinical outcome, and whether UPs can be useful markers for predicting patient prognoses.

Taking advantage of the recent availability of a set of tissue microarrays, we evaluated UP expression in a large cohort consisting of 335 bladder specimens from 285 patients that represent all stages and grades of urothelial carcinomas and different clinical outcome. This allowed us to correlate and systematically analyze the status of UP expression with pathological parameters as well as disease prognoses. Our data indicate that UPs are retained by a significant portion of well-advanced urothelial carcinomas and that persistent UP expression in invasive and metastatic urothelial carcinomas is associated with a favorable prognosis. These findings suggest that UPs can serve as useful, adjunct markers for patient stratification and for predicting clinical outcome of patients with advanced bladder cancers.

MATERIALS AND METHODS

Patient Population

The present study consisted of three patient cohorts: 202 patients who underwent radical cystectomy with bilateral lymphadenectomy; 74 patients who underwent transurethral resection of their bladder tumor (TUR); and nine patients who underwent cystectomy for other diseases with no evidence of genitourinary malignancy. Approval was obtained from the Institutional Review Board (IRB) for the Protection of Human Subjects at University of Texas Southwestern Medical Center.

All 383 consecutive patients admitted for treatment of bladder cancer with radical cystectomy and bilateral lymphadenectomy during the period from 1/1987 through 12/2002 were potential candidates for analysis. Our analyses were limited to patients who had bladder urothelial cell carcinomas. Adequate archival tissue was available for 202 of the 383 cystectomy patients. The study cohort was not different from the whole candidate patient population regarding gender, final pathologic grade, final pathologic stage, lymphovascular invasion, and lymph node status. Histology, grade, stage, and presence of carcinoma in situ (CIS) were confirmed by blinded review of the original pathology slides. The criteria for radical cystectomy included tumor-invasion into the muscularis or prostatic stroma, or Ta, T1, or CIS that were refractory to TUR with intravesical chemotherapy and/or immunotherapy. No patient had distant metastasis at the time of cystectomy. Chemotherapy consisted of cisplatin-based regimens. Comprehensive clinical and pathologic data elements were collected and entered into an IRB-approved database. Multiple data reviews and quality checks were performed to insure the accuracy and completeness of data elements.

The study also consisted of 74 patients who had Ta, CIS, and/or T1 carcinomas on TURBT (from February 1995 to December 2003). Sixty-three patients (85%) had prior TURBTs and

fifty-four patients (73%) were treated with intravesical therapy such as Bacillus Calmette-Guerin (BCG) and mitomycin C. Histology, grade, stage and presence of non-muscle invasive carcinoma were confirmed by blinded re-review of the original biopsy slides. The 1997 TNM classification was used for pathologic staging (25), and the 1973 WHO classification was used for pathologic grading (26). Disease recurrence was defined as any evidence of tumor in a retained bladder at least three months after treatment. Disease progression was defined as the development of invasive urothelial carcinoma ($\geq T1$) when initial diagnosis was CIS or Ta, or the development of muscle-invasive TCC ($\geq T2$) when initial diagnosis was T1. When patients died, the cause of death was determined by the treating physicians, by chart review corroborated by death certificates, or by death certificates alone. Most patients who died of bladder cancer had progressive, widely disseminated, and often highly symptomatic metastases at the time of death. Peri-operative mortality (within 30 days of surgery) was censored for bladder cancer-specific survival analyses. Median age at TUR was 63.2 years (range: 41.1 to 89.3). The median follow-up was 42.3 months (range: 0.3 to 124.6) for those patients alive at the time of analysis.

Tissue Microarray

The tissue microarray comprised: (i) non-cancer tissue from nine patients treated with cystectomy for causes other than malignancy; (ii) tumor tissue from the primary, index cancer from 74 patients treated with TUR; (iii) tumor tissue from the primary, index cancer from 202 patients treated with radical cystectomy; and (iv) lymph node from 50 of the 202 patients treated with radical cystectomy.

H&E-stained sections were reviewed to select representative areas of the index tumor from which to acquire cores for microarray production. The index tumor was defined as the largest and/or highest tumor stage and grade. Tissue microarray blocks were constructed by taking core samples from morphologically representative areas of paraffin-embedded tumor tissues and assembling them on a recipient paraffin block. This was done with a precision instrument (Beecher Instruments, Silver Spring, MD) that uses two separate core needles for punching the donor and recipient blocks and a micrometer-precise coordinate system for assembling tissue samples. For each case, three replicate 0.6 mm core diameter samples were collected and placed on separate recipient blocks randomly, with a 0.5 mm inter-sample space. Multiple internal controls were also placed with the standard controls. Five-micrometer sections were cut from the microarray and stained with H&E to confirm the presence of tumor and to reassess the tumor histology.

Sample tracking was based on coordinate positions for each tissue spot in the tissue microarray block; the spots were transferred onto tissue microarray slides for staining. This sample tracking system was linked to a Microsoft Excel database containing demographic, clinical, pathologic, and survival data on each patient. The array was read according to the given tissue microarray map; each core was scored individually, and the results were presented as the mean of the three replicate core samples.

Pathology

Staff pathologists with expertise in genitourinary pathology examined all specimens according to previously published protocols. To ensure validity of the data extraction, two clinicians read pathology reports of 162 consecutive cystectomy patients, while blinded to patient clinical parameters and the finding of the other reviewer. Inter-reader reliability measured using the intra-class correlation coefficient was greater than 0.95 for all pathologic parameters.

Immunohistochemical Staining and Scoring

A pan-uroplakin antibody, originally generated in rabbits against highly purified, native bovine asymmetric unit membranes (AUMs) (12), was chosen for this study. On Western blotting,

this antibody reacted strongly with UPIII, moderately with UPIb and weakly with UPII of the AUMs of several species including the humans. Tissue sections were routinely deparaffinized and hydrated, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 15 min. Antigen retrieval was carried out by microwaving tissue sections at full power for 15 min in 0.01 M citrate buffer (pH6.0). After an overnight incubation at 4°C with the primary antibody (1:20,000 dilution), tissue sections were incubated with a biotinylated goat anti-rabbit secondary antibody. The sections were then incubated with avidin conjugated with horseradish peroxidase and developed in a solution containing 3, 3'-diaminobenzidine (DAB) and H₂O₂. All sections were counterstained with hematoxylin, dehydrated, mounted and observed by light microscopy. Antibody-stained slides were scored after the entire slide had been evaluated. The expression of UPs in each sample was assigned with a proportion score representing the number of positively stained tumor cells (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3; to 2/3; and 5, >2/3), as well as an intensity score representing the average intensity of the positive tumor cells (0, none; 1, weak, 2, intermediate; and 3, strong) (27). These two scores were combined to yield a total score, which ranged from 0 to 8. All slides were scored independently by two investigators blinded completely from patient information.

Immunohistochemical staining was also performed using antibodies against Ki-67, cyclin E1, cyclin D1, p53, p21, pRB, p27, bcl-2, caspase-3 and survivin on serial sections from the same paraffin-embedded tissue microarray blocks (28). Immunoglobulins from un-immunized rabbits and/or non-specific IgG1 monoclonal antibody were used as negative controls. Multiple established positive control sections were included in each run. Immunostaining was carried out on the Dako Autostainer Staining (Carpinteria, CA). Staining and scoring methods were as previously described.

Statistical Analysis

The Chi-square test was used to evaluate the association between UP and clinicopathologic parameters. Chi-square test for trend was used for comparison of ordinal variables. Mann Whitney *U*-test was used to assess the differences in variables with a continuous distribution between dichotomous categories. Kaplan-Meier method was used to calculate survivals, and differences were assessed with the log rank statistic. Univariate and multivariate survival analyses were performed using the Cox proportional hazard regression model. The lowest category was used as the referent category when calculating the hazards ratios. Statistical significance was set as $p \leq 0.05$. All reported p-values are two-sided, and all analyses were performed with SPSS (version 13.0).

RESULTS

Prevalence of Uroplakin (UP) Expression in Different Stages of Urothelial Carcinomas

Of the 335 specimens surveyed, UPs were found to be present in all 9 normal urothelial specimens of the non-carcinoma conditions (100%), 63/74 (85%) patients who had non-muscle-invasive bladder urothelial carcinomas on TUR, 104/202 (51.5%) patients who underwent radical cystectomy for advanced bladder carcinomas and 33/50 (66%) lymph node metastases (Tables 1 & 2). These frequencies were generally in line with those previously reported based on relatively smaller patient cohorts (20), and reaffirmed the concept that UPs, despite being the terminal differentiation markers of the urothelium, can be retained even in some well-advanced urothelial carcinomas.

Aberrant Patterns of Uroplakin (UP) Expression in Urothelial Carcinomas

Consistent with the notion that UPs are synthesized during the terminal stage of urothelial differentiation (12), immunohistochemical staining using a pan-UP antibody showed

predominantly apical surface labeling of normal urothelia in all nine cases studied (Fig. 1A). However, there was clear evidence of aberrant UP labeling even in early-stage urothelial carcinomas such as carcinoma in situ (CIS) and low-grade, superficial papillary carcinomas of various pathological grades (G1–3). In CIS, regions of uniform staining involving all urothelial layers were observed, particularly in areas with denuded apical surface (Fig. 1B, arrowheads). Intense membranous and cytoplasmic staining was also noted in in-grown cellular aggregates (Fig. 1B, arrows), as was an inverted polarity with strong intermediate/basal layer staining and weak apical staining (Fig. 1C). In low-grade, superficial papillary carcinomas, several staining patterns were common, including uniform (Fig. 1D), micro-luminal (Fig. 1E) and basal-laminal staining (Fig. 1F, G & I). Of particular interest was the basal laminal pattern, where even a small collection of cells was surrounded by strong, linear staining of the periphery (Fig. 1F & G, arrowheads). It appears, therefore, that the micro-luminal and basal laminal patterns represent two opposite differentiation polarities, with perhaps more differentiated cells situated in the center of the tumor nests in the micro-luminal pattern and with those cells in the periphery in the basal laminal pattern. Staining with other luminal and basal cell markers should help determine whether this is truly the case. Overall, there was no clear association between a specific staining pattern and a particular pathological grade. UP-negative tumors were also observed (Fig. 1H), although this accounted for only a minor fraction (15%) of all TURBTs (Table 1).

About half of the muscle-invasive carcinomas were UP-positive (represented by Fig. 2A–D). In these UP-positive carcinomas, strong, uniform staining was frequently observed on three different levels: (i) it often involved all tumor nests; (ii) most cells within a tumor nest were strongly stained; and (iii) both membrane and cytoplasm were stained. In another half of the invasive carcinomas, UPs were completely absent (Fig. 2E–F; Table 2). Since the status of UP had no association with any histopathologic pattern, the UP staining outcome could not be predicted without the actual antibody staining. The labeling patterns of lymph node metastases were similar to those of the muscle-invasive carcinomas, with 66% being UP-positive (Fig. 3A–C) and the rest being UP-negative (Fig. 3D).

Association of UP Expression with Characteristics of Patients with Non-muscle-invasive Bladder Urothelial Carcinomas

Of the 74 patients who underwent transurethral resection for the treatment of superficial urothelial carcinomas of the bladder, UP status was not associated with age or gender (Table 1), neither was it associated with tumor grade (G1–3) or stage (Ta, Tis, T1). Tumors recurred in 38/74 patients (51%) and progressed in 21/74 patients (28%). Overall, 24/74 (32%) patients were dead at the time of the analysis. Eight of these 24 patients died of metastatic bladder carcinomas and the remaining 16 died of other causes without evidence of disease progression. With a median follow-up of 42.3 months (range 0.3 to 124.3), Kaplan Meier analyses showed no association between UP expression and disease recurrence, progression, or mortality ($p=0.181$, $p=0.384$, and $p=0.288$, respectively).

Association of UP Expression with Characteristics of Patients with Muscle-invasive Bladder Urothelial Carcinomas

The association of UP expression with clinico-pathologic characteristics of the 202 radical cystectomy patients is shown in Table 2. Absence of UP expression was significantly associated with advanced pathologic stage, lymphovascular invasion, lymph node metastases, and stage grouping ($<pT3\ N0$ versus $\geq pT3\ N0$ versus $pTany\ Npositive$). Median age at radical cystectomy was 66.2 years (range: 34.2 to 88.0). Median number of lymph nodes removed was 17 (range: 10 to 53). Median time from last TUR to radical cystectomy was 1.7 months (range: 0.1–11.3). There were no differences in age at time of cystectomy, time from last TUR to

radical cystectomy, and number of lymph nodes removed at radical cystectomy between UP-positive and UP-negative groups (p-values ≥ 0.265).

Disease recurred in 97/202 patients (48.0%) and 99/202 (49.0%) patients were dead at the time of the analysis. Of these 99 patients, 82 patients died of metastatic bladder cancer, and 17 patients died of other causes without evidence of disease progression. The mean follow-up was 48.6 months (median 36.4, range 1.3 to 176.1) for those patients alive at the time of the analysis. Kaplan Meier analyses revealed that absent UP expression was significantly associated with an increased risk of disease recurrence and bladder cancer-specific mortality (p=0.023 and p=0.022, respectively; Fig. 4). For example, 57.8% of the invasive urothelial carcinoma patients who were UP-positive were still surviving without recurrence 10 years after radical cystectomy, compared to only 29.8% of the same group who were UP-negative (Fig. 4A).

In multivariate Cox proportional hazards regression analyses that adjusted for the effects of tumor grade and lymphovascular invasion, UP expression was not associated with disease recurrence and bladder cancer-specific mortality (p=0.495 and p=0.503, respectively), whereas tumor stage (p=0.002 and p=0.031, respectively) and lymph node metastases (p<0.001 and p<0.001, respectively) were. The same was true in the subgroup of patients with organ-confined disease (<pT3 N0; n=85).

Association of UP Expression with Other Molecular Markers

To determine whether UP status correlates with other molecular markers, we performed immunohistochemical staining using antibodies against cyclooxygenase-2 (COX-2), p27, p53, pRb, p21, cyclin E1, cyclin D1, Bcl-2, survivin, Ki-67 and caspase-3, on serial sections from the same paraffin-embedded tissue microarray blocks. We found that UP expression was not associated statistically with any of the aforementioned markers (p-values ≥ 0.098 ; data not shown). This result established that there is no apparent functional link between UP expression and cell cycle deregulation in urothelial carcinoma cells.

DISCUSSION

Since our identification of uroplakins (UPs) as the major differentiation products of normal urothelium (12), several attempts have been made to determine whether these proteins are also expressed by the urothelial carcinomas (20,21,29). Most of the published studies have evaluated a relatively small number of cases, with inadequate representation of urothelial carcinomas of different pathological grades and stages. In those studies, the frequencies of UP detection varied anywhere from 40% to 90%. In addition, relatively few late-stage, invasive carcinomas that had undergone radical cystectomy have been systemically analyzed. The analysis of such advanced stage carcinomas is important because it could yield more definitive information about urothelial transformation and loss of UP expression than the early-stage carcinomas. Furthermore, clinical characteristics and prognostic data of the previous studies were often unavailable, and consequently the relationship between UP status and patient outcome remains largely unknown.

Taking advantage of the availability of a tissue microarray, we surveyed the largest cohort of urothelial carcinomas thus far for UP expression and interpreted the UP expression data in the context of clinical characteristics, pathological parameters and patient outcomes. Of the 335 specimens from 285 patients studied, UPs were detected in 100% (9/9) of the normal urothelia, 85% (63/74) of the non-muscle-invasive bladder urothelial carcinomas (including Ta, Tis and/or T1) from TUR, 51.5% (104/202) of the invasive carcinomas from radical cystectomy patients, and 66% (33/50) of the lymph node metastases (Tables 1 and 2; Figs. 1–3). These results indicate that, although there is generally a progressive reduction of UP expression during urothelial transformation and tumorigenesis, these proteins do not completely disappear, even

in the most advanced, late-stage carcinomas. In fact, UP retention and/or over-expression are evident in a significant portion (~50%) of such carcinomas (Figs. 2–3). These results are unexpected given the fact that UPs are synthesized primarily in the superficially located, terminally differentiated umbrella cells of the normal human urothelium. The expression of UPs in some, but not in other, invasive urothelial carcinomas has important implications from both biological and clinical standpoint.

First, it raises a biologically intriguing issue as to whether UP expression is subject to different regulations within a seemingly similar set of carcinomas. For instance, those carcinomas that lack UP expression might have lost the regions or the entire chromosomes harboring certain UP genes. Chromosomal deletions, some of which are highly selective, are known to be exceedingly common in invasive urothelial carcinomas (30). It is worth noting, however, that the four major UPs and one minor UP are localized to five different chromosomes (UPIa at 19q13.13; UPIb at 3q13.3–q21; UPII at 11q23; IIIa at 22q13.31; IIIB at 7q11.2) (<http://www.ncbi.nlm.nih.gov/mapview/>). Thus, it would seem unlikely that all the five UP-bearing chromosomes are lost simultaneously as a main cause of absent UP expression. Indeed, UP-bearing chromosomes do not appear to be among the most frequently lost according to the available cytogenetic data (31). In this regard, absence of UP expression may be due to other mechanisms, such as transcriptional silencing. Cowled and colleagues have shown that hypermethylation of the CpG islands within the proximal promoter of UPIb could lead to the loss of its gene expression (32). Less is known about the methylation status of other UPs in urothelial carcinomas. Alternatively, absence of UP expression could occur on a protein level as a result of specific defects in UP assembly, as has been suggested from studies of cultured normal urothelial cells and UP knockout mice. For example, cultured bovine urothelial cells synthesize large amounts of UP mRNAs without making corresponding amounts of UP proteins, probably due to the lack of cytoplasmic vesicles that would otherwise allow UPs to be assembled into two-dimensional crystals (22). In UP knockout mice, the loss of UPII or UPIII leads to a dramatic reduction of their natural partners, UPIa or UPIb, respectively (18). This is consistent with the fact that singly transfected UPs in cultured cells cannot exit efficiently from the endoplasmic reticulum, thus being rapidly degraded (17,22). Thus, the coordinated expression of UPs and their proper post-synthetic assembly may play a critical role in UP stability and immunohistochemical detectability. It will be interesting to find out using antibodies against individual UPs as to whether late-stage, UP-positive carcinomas contain all UPs or specific pairs of UPs or only individual UPs. Reduction of UPs could also be due to the disruption of the Golgi complex, a common feature in urothelial carcinomas (33). The Golgi complex is known to be critical for the biosynthesis of the intracellular discoidal and fusiform vesicles that transport the AUM, and its disruption could therefore affect intracellular UP transport. Finally, the replacement of the apical surface of normal urothelial umbrella cells by microvilli in tumor cells could impair the apical targeting of UPs, making them more unstable and easily degraded.

In contrast to the absence of UP expression, another group of invasive and metastatic carcinomas showed apparent UP overexpression, in both intensity and uniformity (Figs. 2 & 3), as compared with the normal urothelium. The mechanism(s) of why this occurs is unclear, but the induction could be interpreted as a compensatory effect of up-regulating major differentiation products in order to counter the proliferative effects of the tumor cells. This type of compensatory effects is reminiscent of the induction of certain tumor suppressor genes in response to proliferative stresses (34). If this were the case, then the UP-over-expressing carcinomas would be expected to have a more favorable prognosis than that of the UP-negative ones (see below). Another interesting finding of our study is that UP-absent and UP-overexpressing are almost always mutually exclusive phenotypes in a given carcinoma. In other words, all tumor nests in a given tumor either over-express the UPs or they all lose them (Figs. 2 & 3). This probably reflects the clonal origin in a specific tumor of all tumor cells possessing

the same genotypic and phenotypic characteristics. It also raises the interesting possibility that UP-positive and -negative carcinomas might have evolved from different cancer stem cells. Under this scenario, the UP-positive carcinomas might have originated from the superficially located umbrella cells of the urothelium, which normally synthesize large amounts of UPs. There is increasing evidence suggesting that even differentiated cells can be converted into cancer stem cells under certain conditions (35). On the other hand, the UP-negative carcinomas might be derived from cells in the basal and intermediate urothelial layers where UPs are normally absent (12,20). Further experimental work is needed to test this hypothesis.

Second, our current data suggest that the status of UPs can be used as a potential prognostic indicator for patients with invasive and metastatic carcinomas. In univariate, but not in multivariate, analyses, UP negativity is significantly associated with lymph node metastases, advanced disease stage and increased risk of disease recurrence and cancer-specific mortality (Fig. 4). Therefore, UPs belong to the relatively short list of biomarkers that can be used as an adjunct to predict the outcomes of bladder cancer patients after radical cystectomy (36). Interestingly, UP expression was not statistically associated with markers commonly altered in patients with urothelial carcinomas including COX-2, p27, p53, pRb, p21, cyclin E1, cyclin D1, Bcl-2, survivin, Ki-67 and caspase-3 (data not shown). This suggests that there is no significant functional relationship between these markers and UPs. Thus, UP may have a cooperative effect when combined with a panel of established molecular markers for predicting the prognosis of late-stage bladder urothelial carcinomas.

Third, our results reaffirm the usefulness of UPs as excellent markers for identifying carcinomas of the urothelial origin in differential diagnosis. It remains a major challenge for pathologists at times to differentiate extra-vesicle spread of urothelial carcinomas from poorly differentiated tumors originated from the neighboring organs such as prostate, vagina and uterus. Increasingly, organ-specific markers are being used to provide a more accurate differential diagnosis for the genitourinary carcinomas than just by the routine histopathological examination. Because UPs are rather specific for urothelium and its carcinomas, a positive staining would be highly indicative of a urothelial carcinoma, although a negative UP staining does not rule out such a diagnosis. The relatively moderate (~50%) sensitivity of UP detection could be improved considerably by more sensitive detection methods, such as RT-PCR (37). Even a small number of UP-positive tumor cells inside a lymph node in the case of “micrometastases” could conceivably be detected with this method. Indeed, Theodorescu and coworkers recently showed that amplifying UPII mRNA by RT-PCR was capable of identifying additional positive lymph nodes that were previously missed by routine histopathological examinations (38). Additional studies are needed for UPs and other urothelium-specific markers to be applied in the clinics to help better manage patients with the urothelial carcinomas.

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Figure 1.

Uroplakin (UP) expression in normal urothelium and superficial urothelial carcinomas. Cross-sections of normal urothelium (A) and representative lesions of CIS (B & C), Ta (Grade 1; panel D) and T1 (Grade 1–2, panels E–H; Grade 3, panel I) carcinomas were stained with a pan-uroplakin antibody and counter-stained with hematoxylin. Normally associated with the urothelial apical surface (A), UP staining pattern was aberrant (B–G, I) or lost in urothelial carcinomas (H; see Results for detailed description). Magnifications are 200 x for all panels except panels C & G (400 x).

Figure 2.

UP expression in muscle-invasive urothelial carcinomas of the bladder. Representative cases were shown, some over-expressing the UPs (A–D), whereas others were completely negative (E & F). In UP-expressing specimens, frequently all tumor nests expressed these proteins (A–D). This was in stark contrast to the UP-negative specimens where all tumor nests were frequently negative (E & F). Magnifications are the same for all panels (200 x).

Figure 3. UP expression in lymph node metastases of urothelial carcinomas. Representative cases were shown, including strong-expressing ones (A & B), weak-expressing one (C) and negative one (D). All panels were the same magnification (200 x).

Figure 4. Probabilities of (A) recurrence-free survival and (B) bladder cancer-specific survival in 202 patients who underwent radical cystectomy with bilateral lymphadenectomy stratified according to UP expression.

TABLE 1

Association of uroplakin expression with clinico-pathologic characteristics of 74 patients who were treated with transurethral resection for Ta, Tis, or T1 bladder urothelial carcinomas

	No. Pts (%)	Uroplakin expression		P value
		Negative	Positive	
Total (%)	74 (100)	11 (14.9)	63 (85.1)	
Gender (%) [†]				
Female	14 (19)	4 (28.6)	10 (71.4)	0.203
Male	60 (81)	7 (11.7)	53 (88.3)	
Intra-vesical therapy (%) [†]				
Negative	21 (28)	3 (14.3)	18 (85.7)	1.000
Positive	53 (72)	8 (15.1)	45 (84.9)	
TUR tumor stage (%) [‡]				
Ta	26 (35)	2 (7.7)	24 (92.3)	0.187
Tis	13 (18)	1 (7.7)	12 (92.3)	
T1	35 (47)	8 (22.9)	27 (77.1)	
TUR tumor grade (%) [‡]				
Grade 1	7 (9)	0 (0)	7 (100)	0.153
Grade 2	32 (43)	3 (9.4)	29 (90.6)	
Grade 3	35 (47)	8 (22.9)	27 (77.1)	

[†]Fishers exact test.

[‡]Chi square test.

TABLE 2

Association of uroplakin expression in the primary, index tumor of 202 consecutive patients treated with radical cystectomy and bilateral lymphadenectomy for bladder urothelial carcinomas with clinico-pathologic characteristics

	No. Pts (%)	Uroplakin expression		P value
		Negative	Positive	
Total (%)	202 (100)	98 (48.5)	104 (51.5)	
Gender (%) [†]				
Female	42 (20.8)	17 (40.5)	25 (59.5)	0.298
Male	160 (79.2)	81 (50.6)	79 (49.4)	
Pathologic tumor stage (%) [‡]				
Ta, Tis, T1	21 (10.4)	5 (23.8)	16 (76.2)	0.042
T2	64 (31.7)	28 (43.8)	36 (56.3)	
T3	81 (40.1)	44 (54.3)	37 (45.7)	
T4	36 (17.8)	21 (58.3)	15 (41.7)	
Pathologic grade (%) [†]				
Grade 1 or 2	13 (6.4)	5 (38.5)	8 (61.5)	0.570
Grade 3	189 (93.6)	93 (49.2)	96 (50.8)	
Lymph node status (%) ^{*†}				
N0	136 (67.7)	59 (43.4)	77 (56.6)	0.035
N1, N2	65 (32.3)	39 (60.0)	26 (40.0)	
Pathologic stage risk grouping [‡]				
Organ confined disease (<pT3 N0)	75 (37.1)	29 (38.7)	46 (61.3)	0.042
Extravesical disease (≥pT3 N0)	62 (30.7)	30 (48.4)	32 (51.6)	
Lymph node metastases (pTany N1–2)	65 (32.2)	39 (60.0)	26 (40.0)	
Lympho-vascular invasion (%) ^{*†}				
Negative	102 (50.7)	43 (42.2)	59 (57.8)	0.067
Positive	99 (49.3)	55 (55.6)	44 (44.4)	
Concomitant carcinoma in situ (%) ^{*†}				
Negative	116 (57.7)	58 (50.0)	58 (50.0)	0.775
Positive	85 (42.3)	40 (47.1)	45 (52.9)	
Adjuvant radiation therapy [†]				
Negative	182 (90.1)	84 (46.2)	98 (53.8)	0.058
Positive	20 (9.9)	14 (70.0)	6 (30.0)	
Adjuvant chemotherapy [†]				
Negative	145 (71.8)	72 (49.7)	73 (50.3)	0.641
Positive	57 (28.2)	26 (45.6)	31 (54.4)	

* Lymph node status, lympho-vascular status, and carcinoma in situ status were not available in one patient.

[†] Fishers exact test.

[‡] Chi square test.