Identification of Genes Up-Regulated in Urothelial Tumors

The 67-kd Laminin Receptor and Tumor-Associated Trypsin Inhibitor

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Studies investigating changes in gene expression in urothelial carcinoma have generally compared tumors of different stages and grades but comparisons between low-grade, noninvasive tumors and normal urothelium are needed to identify genes involved in early tumor development. We isolated the urothelium from a low-grade tumor and corresponding normal mucosa by laser capture microdissection on frozen sections. The RNA extracted was amplified to generate suppressive subtractive cDNA libraries. Random sequencing of cDNA clones identified ~100 unique species. Of these 83% were known genes, 15% had homology to genes with an unknown function in humans, and 2% did not show homology to any published gene sequence. Two of the known genes, the 67-kd laminin receptor (67LR) and tumor-associated trypsin inhibitor (TATI), had previously been associated with metastatic progression in many tumor types, although 67LR has not been investigated in urothelial tumors. Immunolabeling of the original tissue with antibodies against these two genes confirmed overexpression, validating our strategy: 67LR was not expressed in the normal urothelium but was present in the tumor, whereas TATI expression was confined to umbrella cells in the normal urothelium, but extended to all cell layers in the tumor. We investigated both markers further in a separate series of tumors of different stages and grades. TATI was more consistently overexpressed than 67LR in all tumor grades and stages. Levels of secreted TATI were significantly higher in urine samples from patients with tumors compared to controls. Our strategy, combining laser capture microdissection and cDNA library

construction, has identified genes that may be involved in the early phases of urothelial tumor development rather than with disease progression, highlighting the importance of comparing tumor with normal rather than just tumors of different stages and grades. (Am J Pathol 2003, 163:493–504)

Low-grade, noninvasive urothelial tumors rarely progress to muscle invasive disease but ~70% of these tumors recur.¹ This has led to the development of rigorous protocols for cystoscopic follow-up, because noninvasive urine-screening tests such as cytology have limited sensitivity and specificity and are observer-dependent. Diagnostic kits have been devised to overcome this problem, but perform only slightly better than cytology. For instance, the average published sensitivities and specificities are 60% and 77%, respectively, for the Bard bladder tumor antigen (BTA) test, which is based on the detection of human factor H-related protein, and 67% and 72%, respectively, for the NMP22 test, which relies on changes in nuclear matrix proteins.² The results are even poorer for low-grade tumors with sensitivities of less than 50% for both the Bard test and cytology.³ A better understanding of the events associated with early tumor development may lead to the development of more sensitive and specific tests.

One approach to identifying the genes involved would be to compare gene expression of normal urothelium and tumor tissue. However, most gene expression studies of clinical material have compared tumors of different stages and grades,^{4–6} therefore concentrating on genes involved in tumor progression rather than initiation. One of the reasons for this may be that there is generally sufficient tumor mRNA for direct comparisons, whereas

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normal urothelium is composed of only a few cell layers, with a poor yield of mRNA. Cell lines have been used to circumvent the problem of mRNA abundance.^{7,8} Although findings from tumor cell lines can subsequently be validated on tissue samples and microarrays as elegantly shown by Sanchez-Carbayo and colleagues,⁹ this strategy cannot be used for direct comparisons with normal cells, as many of the genes they express *de novo* in culture are also expressed by tumor cells.¹⁰ A further difficulty in the use of fresh clinical material is that the morphological detail on frozen sections may be too poor to confidently distinguish between normal urothelium and low-grade dysplasia, a distinction that is difficult even on optimally fixed material.^{1,11–13} Finally, normal and dysplastic urothelium are often intermingled, hence the need for selective dissection. We therefore sought to develop a strategy to overcome these difficulties.

We exploited the differential expression of cytokeratin 20 (CK20) in normal *versus* dysplastic urothelium^{14–16} to objectively define areas of normal urothelial organization and maturation. Although CK20, a marker of terminal differentiation, can be negative in highly inflamed or regenerative urothelium, its pattern of expression in stable urothelium is consistent.¹⁵ Superficial umbrella cells are positive and expression is also seen in occasional cells located in the intermediate layers. The latter are tear-shaped, tapering into a thin CK20-positive process extending toward the basement membrane, as is seen for superficially located umbrella cells (personal observation). This contrasts with the full-thickness expression seen in dysplasia/carcinoma *in situ* and in a high proportion of tumors,^{14,15} although a small proportion of papillary tumors may be negative.^{14,16} Using CK20-labeled slides as a reference, normal urothelium was isolated from the adjacent hematoxylin and eosin (H&E)-stained frozen sections using laser capture microdissection.¹⁷ Tumor was identified by its papillary architecture as well as full-thickness CK20 expression. RNA was extracted and amplified and subtractive cDNA libraries were generated. Laser capture microdissection has been used to select tissues for analysis by DNA and cDNA microarrays,^{18–22} or high-density oligonucleotide arrays²³ in other tumor types but we believe that this is the first report combining subtractive hybridization with laser capture microdissection.

The expression of two of the genes that we identified by this method, the 67-kd laminin receptor (67LR) and tumor-associated trypsin inhibitor (TATI), was investigated at the protein level in our test case to validate our approach. We also examined a panel of urothelial tumors of different grades and stages for patterns of tissue expression. Finally we compared tissue expression of TATI to its level in urine, to further explore its potential as a noninvasive marker of disease.

Materials and Methods

Frozen Tissue Preparation

Human bladder specimens were obtained from 10 patients after informed consent. In theater, the specimens

were orientated and mounted on metal disks in cryogenic embedding compound (Leica, Milton Keynes, UK), frozen on dry ice and stored in liquid nitrogen. Serial sections (9 μ m) were prepared using a cryostat with a clean steel blade onto Superfrost plus glass slides. The slides were transferred immediately to -20°C , where they were kept desiccated and used for laser capture microdissection within a few days.

The sample selected for subsequent analysis was from a patient who was presenting for the first time, and had therefore not received any previous intravesical treatment, and whose sample contained the greatest amount of normal urothelium and a well-defined World Health Organization (1999 classification²⁴) low-grade, World Health Organization/International Society of Urologic Pathology (ISUP) (1998 classification²⁵) grade 1 papillary tumor. CK20 expression was restricted to the superficial cells in the normal urothelium, but the tumor showed full-thickness expression.

Staining of Sections for Laser Capture Microdissection

Rapid immunostaining protocols for use with laser capture microdissection have been developed.²⁶ However because of the anticipated low yield of mRNA from normal urothelium and the concern about compromising its quality, sections for dissection were stained by a rapid method for H&E only, but every fifth section was labeled for CK20 to distinguish areas of normal and dysplastic urothelium.

For H&E staining, all solutions were prepared using autoclaved diethyl pyrocarbonate-treated (0.1% v/v) double-distilled water. Thawed sections were immersed in 70% ethanol for 30 seconds, rinsed in diethyl pyrocarbonate-treated water, stained in Meyer's hematoxylin for 2 minutes, washed in diethyl pyrocarbonate-treated water for 30 seconds and blued in Scott's solution [2% (w/v) MgSO_4 and 0.35% (w/v) $\text{Na}_2\text{H}_2\text{CO}_3$] for 1 minute. Tissues were dehydrated through 75% and 95% ethanol (30 seconds each) and counterstained in eosin for 2 minutes. Finally, sections were washed for 1 minute in two changes each of 95% ethanol, absolute ethanol, and xylene, and air-dried.

Frozen sections were labeled for CK20 expression. Briefly, sections were fixed in acetone for 2 minutes and air-dried. Endogenous avidin-binding activity was quenched using an avidin-biotin blocking kit (Vector Laboratories, Peterborough, UK), according to the manufacturer's instructions. Sections were incubated for 10 minutes with normal rabbit serum (10% v/v in Tris-buffered saline) to block nonspecific secondary antibody binding. Sections were incubated with primary antibody (clone CK20.3; LabVision, Newmarket, UK) for 60 minutes, followed by incubation with biotinylated rabbit anti-mouse immunoglobulins (DAKO, High Wycombe, UK) for 30 minutes. Finally, sections were incubated for a further 30 minutes with the streptavidin-biotin-horseradish peroxidase complex (ABC kit, DAKO), prepared according to the manufacturer's instructions. Sections were washed

with Tris-buffered saline, pH 7.6, after each step and all incubations were performed at ambient temperature in a humidified atmosphere. Antibody binding was visualized after a peroxidase-activated 3,3'-diaminobenzidine (DAB; Sigma, Gillingham, UK) substrate reaction for 15 minutes and counterstained with hematoxylin, dehydrated, cleared, and mounted in DePeX.

Laser Capture Microdissection

Microdissection was performed using a PixCell II laser capture microscope equipped with an infrared diode laser (Arcturus Engineering, Mountain View, CA), as described by others.²⁶⁻²⁸ The laser power was 60 mW, the pulse width was 50 ms, the spot size was 30 μ m, and in total ~70,000 laser shots were needed to collect the RNA from 20 slides. Two strategies were followed. Either the selected areas were microdissected from the tissue, or where the area of interest made up the majority of the section, it was found most efficient to dissect and discard areas that were not of interest. In the latter case, sterile 25 μ l "Easiseal" frames and coverslips (Hybaid Ltd., Ashford, UK) were used to encapsulate the tissue sections from which the RNA could then be extracted.

RNA Extraction

The RNA from laser-captured material was extracted using the Purescript RNA isolation kit by pipetting the lysis solution directly on to the caps, pipetting up and down, transferring the product to a microfuge tube, and then following the manufacturer's recommended protocol (Flowgen, Lichfield, UK). RNA quality was assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers for β 2 microglobulin, which amplify a fragment of 123 bp (forward 5'-CTCGCGCTACTCTCTCTTCT-3' and reverse 5'-TGTCGGATTGATGAAACCCAG-3'). Each RT-PCR reaction was performed using RNA from an individual tissue section. Whole ureter RNA was used as a positive control. To prepare cDNA, 120 ng of tumor and normal RNA was heated in diethyl pyrocarbonate-treated water containing oligo dT and RNasin at 65°C for 5 minutes, 37°C for 10 minutes, and cooled on ice. A reaction buffer containing PCR buffer II (Perkin Elmer, Warrington, UK), 8 mmol/L MgCl₂, 0.5 mmol/L dNTP, 1 pmol each of forward and reverse primers and 5 U MMLV reverse transcriptase (Pharmacia Biotech., St. Albans, UK) was added to the mix, which was incubated at 37°C for 60 minutes before denaturation at 75°C for 5 minutes. The conditions for the β 2-microglobulin PCR were 96°C for 10 minutes, followed by 33 cycles of 96°C for 30 seconds, 60°C for 1 minute, 74°C for 1 minute, and a final extension of 74°C for 7 minutes, in the presence of 1.25 U *Taq* polymerase (Perkin Elmer). RT-negative controls, from which the RT was omitted, were included in all reactions.

RNA Quantitation

Quantification of RNA was performed by Northern blot analysis because this was highly sensitive and allowed

conservation of the small samples. Approximately one-tenth of the total RNA from the laser-dissected tumor samples was electrophoresed in 1% (w/v) agarose gels containing 1.8% (v/v) formaldehyde and examined on a UV transilluminator to check for RNA degradation. As a standard, RNA prepared from a cell line was quantified on a spectrophotometer and serial dilutions were analyzed on the same agarose gel as the laser-dissected RNA. The RNA was transferred to a nitrocellulose membrane (Hybond N+ membrane; Amersham, Slough, UK) by capillary transfer and hybridized with a riboprobe complementary to 18S ribosomal RNA (Ambion, distributed by AMS Biotechnology Ltd., Abingdon, UK), which had been labeled with [α -³²P] CTP (Amersham) using the T7 labeling kit (Promega, Southampton, UK). The Rapid-hyb system (Amersham) was used for hybridization according to the manufacturer's instructions and the amount of hybridized probe was quantified on a phosphorimager (Molecular Imager FX System; Bio-Rad Laboratories, Hemel Hempstead, UK).

Construction of Subtractive cDNA Library

One hundred ng of total RNA was used to prepare cDNA using the SMART cDNA synthesis amplification kit (Clontech, Basingstoke, UK). The RNA was not DNase-treated before the production of cDNA to minimize RNA loss and as the SMART cDNA method utilizes primers specific for mRNA. Subtractive hybridization was performed using the PCR Select Subtractive Hybridization kit (Clontech) based on the method of suppression subtractive hybridization developed by Diatchenko and colleagues.²⁹ This method selectively amplifies differentially expressed sequences, and the generation of high- and low-abundance sequences is equalized during the first hybridization. The PCR allows amplification of equalized differentially expressed sequences. Each step of the cDNA synthesis and subtractive hybridization procedure was monitored using the positive control samples provided by the manufacturer. We verified the efficiency of subtraction by PCR analysis by comparing GAPDH levels in subtracted and unsubtracted cDNA using the method and GAPDH primers provided by the manufacturer.

cDNAs generated were cloned into the pGEM-T Easy vector using a ligation kit (Promega) and transformed into JM109 competent bacteria. Colonies selected at random were grown overnight and used to prepare plasmid DNA (Qiagen, Crawley, UK). The presence of an insert within the plasmid DNA was assessed by digestion using *Eco*RI and analyzed in 1% agarose gels. Sequencing of plasmid DNA insert was performed on an ABI Prism 377 sequencer using Big Dye terminator sequencing (Perkin Elmer). Each sequence was compared to the EMBL database.

Immunohistochemical Validation

Twenty-eight tumors from 26 patients were investigated for the expression of 67LR and TATI. These patients had also given consent for urine testing. There were 18 non-

invasive tumors (pTa grade 1, $n = 4$; grade 2, $n = 11$; grade 3, $n = 3$), 6 tumors invading the lamina propria (pT1 grade 2, $n = 2$; grade 3, $n = 4$), and 4 muscle invasive tumors, all of grade 3. Seven of the patients with noninvasive tumors and all of the patients with invasive tumors had no previous history of bladder cancer. Biopsies from 11 patients with no history of bladder cancer were also investigated. These patients had presented either with microscopic hematuria or symptoms of bladder dysfunction (we did not have ethical approval to perform biopsies in healthy volunteers). Four had histologically normal bladder mucosa, and the remainder had varying degrees of inflammation with Von Brunn's nest formation, cystitis cystica, or nonkeratinizing squamous epithelium.

The monoclonal antibody clone MluC5 recognizes 67LR.³⁰ The monoclonal antibody against TATI does not cross-react with epidermal growth factor, with which TATI shares genomic and sequence homology.³¹ Immunostaining for CK20 (monoclonal clone 20.8, 1:20; DAKO) was performed to identify areas of normal urothelial differentiation in the flat mucosa as previously described.^{14–16} As these were paraffin sections, trypsinization (10 minutes) was required for antigen retrieval.

Immunohistochemistry on paraffin-embedded tissue was performed as previously described.^{32,33} Briefly, 5- μ m sections were dewaxed and rehydrated. Antigen retrieval was not required for MluC5 but was achieved by microwaving in 10 mmol/L of citrate buffer, pH 6.0, for 10 minutes for TATI. This was followed by blocking endogenous avidin-binding sites using an avidin/biotin blocking kit according to the manufacturer's instructions (Vector Laboratories). Sections were incubated sequentially in primary antibody (MluC5, 1:2000; TATI, 1:1000) biotinylated rabbit anti-mouse IgG secondary antibodies, followed by streptavidin/horseradish peroxidase ABC complex according to the manufacturer's instructions (DAKO), with washing between each step. Negative controls were performed by omission of the primary antibody. Positive controls were a malignant melanoma for MluC5 and duodenal mucosa for TATI. However, normal ureter was subsequently used (as for CK20), once the consistency of TATI expression in normal urothelium was established.

Urine Testing for TATI

Urine was collected before cystoscopy and tumor resection in 24 of the 26 patients. Urine samples were also collected from seven healthy volunteers and four patients with urinary tract infections or stones. Samples were placed on ice and a Complete Protease Inhibitor Cocktail tablet added. After sieving through a 100- μ m nylon filter to remove cell debris, samples were centrifuged for 10 minutes at $800 \times g$ at 4°C and the supernatants aliquoted and stored at –80°C until assayed.

Soluble TATI concentrations were determined using a commercially available radioimmunoassay according to the manufacturer's instructions (Orion Diagnostica, Espoo, Finland). Urinary protein and creatinine concen-

trations were measured by standard automated analysis using a Synchron LX-20. Both TATI and total protein concentrations were adjusted for creatinine concentration and expressed as a TATI/creatinine index or protein $\times$ 1000/creatinine index respectively.

Statistical Analysis

Statistical analysis was performed using the Graphpad Prism Package (GraphPad Software, Inc., 1998–1999, San Diego, CA). As the data were not normally distributed, the nonparametric Mann-Whitney test and Spearman's rank correlation test were used.

Results

Laser microdissection allowed clean capture of normal and pTa G1 urothelium. Comparison against CK20-immunolabeled sections allowed areas of normal (as opposed to dysplastic) urothelium to be identified with confidence. The quality of RNA was sufficient to allow amplification of a 123-bp fragment of β 2-microglobulin by RT-PCR and hybridization of 18S probe to the Northern blot clearly demonstrated an intact 1.8-kb transcript present. The RNA yield was quantified by Northern blot, and varied from 80 ng to 800 ng per 30 tissue sections, depending on the size of the section and the relative area of interest. The efficiency of the subtraction was confirmed by demonstrating lower levels of GAPDH in the subtracted cDNA (Figure 1).

A library containing cDNA putatively up-regulated in tumor was generated. Sequencing of $\sim$ 100 unique clones after random selection from the library revealed 17% coding for genes involved in cellular metabolic processes, including lactate dehydrogenase and cytochrome oxidase IV; 44% involved in translation, many of which were ribosomal proteins; 5% coding for structural proteins; and 17% involved in cell signaling, cell growth, and cell transport (Table 1). The remaining genes either encoded proteins with an unknown function in humans (15%) or did not show homology to any published gene sequence (2%). The finding of up-regulation of two of the known genes, TATI and 67LR, was intriguing as they had previously been associated with disease progression in different cancers, whereas our study was based on early disease. They were therefore selected for further study by investigation at the protein level, first in the tissues from which RNA had been extracted, and then in a larger group of patients to determine whether the results were specific to our test case or extended to a wider population.

Immunocytochemistry for 67LR

In the material from which RNA was isolated, normal urothelium was negative (data not shown) for 67LR whereas there was widespread positivity of basal and intermediate cell layers in the tumor urothelium (Figure 2b). Stromal vessels were also positive. In the larger group of bladder cancer patients, there was no evidence

Figure 1. PCR analysis of subtraction efficiency. **a:** GAPDH band is seen after 18 cycles in the unsubtracted cDNA. **b:** GAPDH band appears five cycles later in the subtracted material, five cycles representing ~20-fold difference in levels, as described by the PCR Select subtractive hybridization kit method (Clontech).

of 67LR expression in 7 of the 12 samples of normal urothelium. Membrane positivity of basal cells was focally present in the remaining five samples (Figure 2a). Of the 18 papillary noninvasive tumors, 12 were positive and 6 negative. The distribution of expression was variable in the tumors, often extending to intermediate and superficial cells rather than confined to basal cells. Positivity was also seen in a proportion of the noninvasive components of invasive tumors (six of nine). Invasive foci were positive in 8 of 10 patients, with both cytoplasmic and membrane positivity (Figure 2c).

The expression of 67LR was very variable in the biopsies of patients with nonneoplastic bladder conditions, although the vessels were consistently positive. In the histologically normal biopsies, there was either no expression in the urothelium, scattered membrane-positive basal cells, or consistently positive basal cells with occasional positive intermediate and even superficial cells. The pattern varied in different areas of the same biopsy. This was also the case in the inflamed biopsies, and expression did not seem to relate to the degree of inflammation. In one biopsy with mild inflammation, there was strong and consistent positivity for basal and intermediate cells, including in Von Brunn's nests and cystitis cystica. In the most severely inflamed biopsy, there was only focal basal expression. In one case, occasional superficial cells were positive. This inconsistent pattern was also observed in the foci of squamous epithelium, with variable basal positivity.

Immunocytochemistry for TATI

The tissue from which the RNA was isolated showed that TATI expression was confined to the terminally differentiated superficial cells in normal urothelium, and absent from the basal and intermediate cell layers (Figure 3a). No staining was present within the underlying stroma. In the papillary tumor, all cell layers or scattered cells throughout the urothelium were positive for TATI, and trapped secretions were also positive (Figure 3b). In our larger series of bladder tumor patients, both normal urothelium and tumor had been sampled in 12 of the 28 cases. In two of the cases of normal urothelium, there was no demonstrable TATI expression, and in the remainder only superficial cells were positive. Dysplasia in flat mucosa showed full-thickness expression (Figure 3c). Of the 18 noninvasive papillary tumors, 1 was negative (as was the normal urothelium in this patient), 1 showed superficial expression only as in the normal urothelium, but 16 showed increased expression ranging from positivity in all urothelial cell layers throughout the tumor to more patchy expression, either confined to certain areas of the tumor or to basal and/or intermediate cell layers. Invasive foci were positive in 9 of the 10 cases (Figure 3d). Expression of TATI was assessed semiquantitatively as either negative, superficial only, variable, or consistently positive throughout the tumor, to allow comparisons of tissue expression with urine concentration.

In the group of patients with nonneoplastic bladder conditions, TATI expression was consistently confined to superficial and occasional intermediate cells when present, and was absent in two cases of severely inflamed urothelium. There was no evidence of expression in areas of squamous epithelium, and Von Brunn's nests were negative. The luminal cells of cystitis cystica were occasionally positive.

Correspondence between CK20, TATI, and 67LR Immunostaining

In our test case, the dysregulation of TATI expression mirrored that of CK20 with full-thickness expression. We therefore compared their patterns in our panel of noninvasive tumors. Although 15 of 18 (83%) tumors showed an abnormal distribution of both markers, the extent and distribution of the positivity were not directly comparable, with some areas negative for one marker and positive for the other. The final three cases were completely negative for one marker (two of three, CK20; one of three, TATI) and positive for the other (Figure 4). None of the tumors were negative for both. The pattern of 67LR did not appear to show any correlation with CK20 or TATI expression.

Urine Concentrations of TATI

The median TATI value was 36.6 $\mu\text{g/L}$ (range, 0 to 249.5) in the bladder tumor patients and 17.7 $\mu\text{g/L}$ (range, 5.1 to 39.0) in the controls ($P < 0.02$). This difference remained significant after correcting for dilution/concentration ef-

Table 1. Genes Identified by Subtractive Suppressive Hybridization between Normal Urothelium and Low-Grade WHO Grade 1 Papillary Tumour, Grouped According to Function

Identity	GenBank accession number	Numbers of clones found
Translation		
Ribosomal protein L5 and S100P Ca binding protein	NM 005980	1
Ribosomal protein S6	NM 001010	3
Elongation factor 1a	M29548	4
Ribosomal protein S13	NM 001017	1
Ribosomal protein S15a	NM 001019	1
Ribosomal protein S23	NM 001025	1
Ribosomal protein L3	NM 000967	2
Elongation factor 2B2	NM 014239	1
Ribosomal protein S3a	NM 001006	1
Ribosomal protein large PO	NM 001002	1
Ribosomal protein S17	NM 001021	2
Ribosomal protein S1	NM 001020	1
Eukaryotic translation initiation factor 4a	NM 001967	1
16S rRNA	J01415	7
Seryl tRNA synthase	NM 006513	1
Eukaryotic translation initiation factor 3	NM 003757	1
RPL10/Wilms tumour related protein	M64241	1
12S rRNA	J01415	2
Ribosomal protein S8	NM 001012	1
Ribosomal protein S13a	NM 012423	1
Ribosomal protein L31	NM 000993	1
Ribosomal protein L37	NM 000997	1
Ribosomal protein S10	NM 001014	1
Ribosomal protein S26	X69654	1
Ribosomal protein L41	NM 021104	1
Ribosomal protein L9	NM 000969	3
Ribosomal protein S27A	NM 002954	1
Ribosomal protein L35a	NM 000996	1
Ribosomal protein L13a	NM 012423	1
Eukaryotic elongation factor 1G	NM 001404	2
Ribosomal protein L23	X55954	2
Ribosomal protein L19	S56985	1
Ribosomal protein S11	NM 001015	1
Ribosomal protein S3	S42658	4
Ribosomal protein S27	NM 001030	1
Ribosomal protein S4	NM 001007	1
Ribosomal protein L17	NM 000985	1
Ribosomal protein S24	NM 001026	1
Ribosomal protein L7	XM001450	1
Ribosomal protein L44	XM010209	1
18S rRNA	K03432	2
Ribosomal protein S7	NM 001011	1
Ribosomal protein S25	XM001028	1
Ribosomal protein S29	XM007282	1
Heterogenous nuclear ribonucleoprotein A1	NM 002136	1
Metabolic processes		
Cytochrome oxidase subunit 1	NC 001897	3
Cytochrome b	AF254896	4
NADH dehydrogenase subunit 4	NC 001807	4
NADH dehydrogenase subunit 2	AF014900	1
Cytochrome oxidase subunit II	J01415	3
NADH dehydrogenase 1	J01415	2
Cytochrome c oxidase subunit VIb	X54473	1
NADH dehydrogenase subunit 3	J01415	1
ATP synthase subunit c	NM 005175	1
Cytochrome c oxidase subunit VIIa	NM 001865	1
Cytochrome c oxidase IV	NM 001861	1
Lactate dehydrogenase	Y00711	2
ATPase 6	V00662	2
F1-ATPase epsilon	AF052955	1
Glutathione peroxidase 2	NM 002083	2
Monoamine oxidase A	M68840	1
Ubiquinone	NM 002495	1
Cytochrome c oxidase subunit III	NC 001807	2
Structural proteins		
Gamma actin	NM 001614	1
Histone H3.3	Z48950	1

(Table continues)

Table 1. *Continued*

Identity	GenBank accession number	Numbers of clones found
Histone H2b	AJ223352	1
ARC34	NM 005731	2
Nonmuscle myosin light chain	M22918	1
Cell signalling, cell growth, cell transport		
Heat shock protein 90	AF028832	1
GNB2L1 G protein	NM 006098	1
RNA-binding protein	AF021819	1
Calmodulin	U94728	1
VDUP1	NM 006472	1
A100a6 Ca binding protein	NM 014624	2
Interferon induced protein	X03557	1
Cdc like kinase 1	NM 004071	1
GABA receptor-associated protein	NM 007278	2
Organic cation transporter	NM 003060	1
Laminin receptor 1	NM 002295	2
PSTI/TATI	M11949	2
C2F	U72514	1
HLA class I heavy chain	X58534	1
Proteasome subunit-gene psmc6	NM 002806	1
Proteasome subunit-gene psmb1	NM 002793	1
DRG 1	NM 004147	1
Ku antigen	M30938	1
Adenosine deaminase	U75503	1
Homology to gene/protein-function unknown		
FAU1	X65921	2
CGI-204	NM 020409	2
Kiaa0924 protein	NM 014897	1
ERH	NM 004450	1
Cgi-134 protein	AF151892	1
Kiaa0543	AB011115	1
HSPC20	AF077206	1
XAG-2	AF038452	1
protein FLJ10883	XM054302	1
DPY-30 like sequence	AF226998	1
Beacon	AF318186	1
Alpha gene	AF203815	1
Chromosome 17	AC006487	
	T68659	1
	AC010227	1
	BE404214	2

fects because the median TATI/creatinine index was 4.45 (range, 0 to 21.5) in the bladder tumor patients and 2.1 (range, 1.35 to 5.6) in the controls ($P < 0.02$), although there was considerable overlap at the lower end of values (Figure 5b). The protein concentration was also higher ($P < 0.002$) in the bladder tumor patients (median, 0.225 g/L; range, 0.01 to 1.54) compared with controls (median, 0.07 g/L; range, 0.01 to 0.33). The most significant difference was found when comparing the protein/creatinine index between the two groups (median, 226.22; range, 61.73 to 2735.3 *versus* 66.67; range 23.81 to 458.33; $P = 0.0006$ in controls) (Figure 5a).

In the bladder tumor patients, there was no correlation between urinary levels of TATI and expression in the tissues using our semiquantitative assessment. For instance, TATI was undetectable in the urine of one patient whose tumor was very strongly and extensively positive. There was no correlation between urinary TATI/creatinine index and grade, but the correlation with stage was significant ($P < 0.05$), although we were unable to correct for the effect of tumor size. There was a highly significant

correlation between the protein/creatinine index and tumor grade ($P = 0.001$) and stage ($P = 0.0002$).

Discussion

Previous reports of gene expression in urothelial carcinoma have generally either used cell lines,⁷⁻⁹ or compared tumors of different stages and grades,⁴⁻⁶ primarily to circumvent problems of mRNA abundance and the difficulties of selecting specific cell populations. Although these studies have highlighted genes of potential relevance to disease progression, they were not designed to identify those involved in the earliest steps of tumor formation. Our study was based on material that was carefully characterized through the application of CK20¹⁴⁻¹⁶ to identify normal urothelium. Both normal urothelium and low-grade tumor were selectively captured and we have shown that it is possible, after an amplification stage, to generate suppressive subtractive cDNA libraries, which are enriched for genes overexpressed in tumor. Micro-

Figure 2. 67LR expression in normal urothelium and tumors. **a:** Example of normal urothelium showing occasional positive basal cells (the test case was negative). **b:** More widespread, but still predominantly basal expression in a noninvasive papillary tumor. **c:** Membrane and cytoplasmic expression in invasive foci contrasting with the absence of expression in residual Von Brunn's nests. Original magnifications, $\times 200$.

Figure 3. TATI expression in normal urothelium and tumors. **a:** Expression predominantly along the luminal edge of umbrella cells in normal urothelium. **b** and **c:** Full thickness, cytoplasmic expression in papillary tumor (**b**), and dysplastic urothelium (**c**). **d:** Cytoplasmic expression in invasive tumor foci. Original magnifications, $\times 200$.

Figure 4. Comparison of CK20 and TATI expression. **a:** The papillary tumor is negative for CK20 although the adjacent normal urothelium shows expression in superficial cells. **b:** The same tumor shows strong full thickness TATI expression with positivity along the luminal edge in the normal urothelium. Original magnifications, $\times 50$.

dissection has been used previously to isolate RNA for the construction of cDNA libraries,^{34,35} and as the starting material for microarray probes,³⁶ but, to our knowledge, has not been used previously for the production of subtractive cDNA libraries. Through this strategy, we have identified both known genes and a number of un-

known sequences, which we are currently investigating further.

Some of the known genes have previously been associated with bladder cancer, providing some corroboration for our approach. These include lactate dehydrogenase^{37,38}, cytochrome oxidase IV,⁶ heat shock protein 90,³⁹ and TATI (synonyms pancreatic secretory trypsin inhibitor, kazal and SPINK1).^{40,41} For instance, overexpression of cytochrome oxidase was found in locally advanced (stages pT2 to pT4) and high-grade disease compared with low-grade, noninvasive tumors, which led the authors to conclude that this enzyme was associated with the development of an aggressive phenotype. This illustrates the value of comparing low-grade tumor with normal epithelium in unraveling the steps in tumor development and progression, because our data show that enzyme levels are already elevated in early tumorigenesis. Therefore, although expression levels may continue to rise during the process of dedifferentiation and invasion, accounting for the earlier findings, our data suggest that this enzyme is not necessarily an initiating factor in the disease process.

The 67-kd laminin receptor (67LR) has been investigated in a variety of tumor types, but not to our knowledge in urothelial tumors. 67LR is a nonintegrin protein, which is co-expressed and co-regulated with the $\alpha 6$ integrin subunit, and is physically associated with this integrin on the cell membrane.⁴² This suggests that it is involved in regulating or stabilizing the interaction of laminin with the $\alpha 6 \beta 4$ or $\alpha 6 \beta 1$ integrin. It has been associated with the metastatic phenotype and poor prognosis in a variety of tumor types.^{43–46} In breast tissue, normal myoepithelial cells consistently express 67LR, whereas luminal cells are negative. However, luminal cells became positive with the development of atypia and *in situ* carcinoma, and all invasive carcinoma show at least focal positivity.⁴⁷ In our study, the findings were not so consistent. The case that served as the starting material for our

Figure 5. a: Comparison of urinary total protein/creatinine index between patients and controls. **Lines** represent median values. Note the logarithmic scale. **b:** Comparison of urinary TATI/creatinine index between patients and controls. **Lines** represent median values.

investigation showed no expression in the normal urothelium, whereas all layers of the papillary tumor were positive, confirming the overexpression found by subtractive hybridization and providing some validation of our experimental approach. In our larger series, 67LR expression was found in 67% of noninvasive tumor components, and again was not confined to basally located cells. This loss of association with the basement membrane was also seen in some samples of normal urothelium from patients with a variety of nonneoplastic bladder conditions, in which expression patterns varied markedly, both between patients and within different areas of the same biopsy. This suggests that 67LR has a function other than adhesion to laminin in urothelial cells. 67LR derives from the dimerization of a 37-kd precursor, which is highly conserved and functions as a ribosomal protein. Construction of a phylogenetic tree shows that it is maintained during the evolution from prokaryotes to eukaryotes and its laminin-binding capability is an additional function acquired during evolution by vertebrates.⁴⁸ Up-regulation of 67LR expression may therefore be related to its ribosomal function and the need for increased protein synthesis for repair of normal urothelium or tumor growth. Expression was also found, at least focally, in 80% of invasive carcinoma, possibly because 67LR also has a function in cell motility. Proteolytic cleavage of extracellular matrix is required to convert it into a substrate suitable for migration of epithelial cells⁴⁹ and protease recognition domains on laminin 1 are conformation-dependent.⁵⁰ Indeed, exposure of laminin to 67LR induces conformational changes in laminin and increased laminin degradation rates by proteases. Tumor cells seeded on 67LR modified protease-cleaved laminin, formed pseudopodia and were motile, whereas those seeded on unmodified cleaved laminin failed to adhere and migrate.⁵¹

TATI was first described in the urine of patients with ovarian cancer.⁵² Although the function of TATI in cancer has not been elucidated, elevated levels have been detected in the serum and urine of patients with bladder cancer in one study, although the stages and grades of the tumors were not stated.⁴¹ Serum values of TATI have also been used in patients with muscle invasive and metastatic transitional cell carcinoma, to monitor response to therapy.⁴⁰ However, although TATI expression has been investigated in normal fetal and adult tissues and was demonstrated in the urothelium of the renal pelvis and bladder,⁵³ its patterns of expression have not been described in urothelial tumors. In our original sample, TATI expression was confined to superficial cells in the normal urothelium but was seen throughout the tumor urothelium and in secreted material within intracytoplasmic lumina. Investigation of a large number of biopsies of nonneoplastic urothelium showed that TATI expression was consistently limited to the superficial, terminally differentiated and occasional intermediate cells, with the exception of a small proportion of heavily inflamed samples, which were negative. Expression was predominantly seen on the luminal edge of umbrella cells, suggesting a role in protection from urinary proteases. In

contrast, a large proportion of noninvasive and invasive tumors showed overexpression.

Urine levels were elevated in patients with bladder cancer compared with controls, although there was considerable overlap toward the lower end of the scale. There was no apparent correlation between extent of tumor expression and urine levels, perhaps because of the additional influence of secretion rates, or rates of cell shedding or cell death. Loss of cell cohesion and consequent shedding is one of the characteristics of poor differentiation, and therefore we expected higher TATI levels in patients with high-grade tumors. This was not the case, although there was a correlation with urine protein levels and tumor grade. Both TATI and protein levels correlated with tumor stage, but this may have been because of tumor volume as more advanced tumors tended to be larger. The best separation between bladder cancer patients and controls was achieved by determination of the protein/creatinine index rather than the TATI/creatinine index. The presence of proteinuria in patients with urothelial tumors is well documented^{54–59} and could be because of the proliferative and metabolic activity of tumor cells, although a host response to the tumor may also contribute to the urinary protein levels.^{54,60} The potential of this test in the diagnosis or follow-up of patients with urinary tract tumors has not been explored, presumably because proteinuria is also present in patients with a variety of other conditions, such as infection or stone disease.⁶¹ Nevertheless given the widespread availability of the test and its low cost, its value in the triage of patients known to be at risk of recurrent tumors merits further investigation. Although a high level may not distinguish between tumor and infection or stones, a low reading may be sufficient to avoid or delay the need for a check cystoscopy, allowing urologists to concentrate resources on high-risk patients.

Although total protein measurement may have greater value than TATI in urine testing, the tissue distribution of TATI may be useful in diagnostic histopathology, because both dysplastic urothelium and papillary tumors appear to show full thickness expression rather than the restricted expression seen in normal urothelium. This change in the localization of expression in neoplastic transformation appears to mirror that seen with CK20.^{14–16} However, further studies are needed to determine whether investigation of TATI expression is of similar or greater diagnostic or prognostic value than CK20.

We have shown that small amounts of RNA extracted from laser-captured microdissected tissues can be used to generate subtractive suppressive cDNA libraries and that this is an effective way of identifying differentially expressed genes from well-defined normal urothelium and urothelial tumors. We were able to confirm our findings by performing immunocytochemistry for two genes, 67LR and TATI, both of which showed more widespread expression in most tumors compared with normal. Both of these gene products had previously been associated with tumor progression but our findings suggest that up-regulation occurs early in tumor development. This illustrates the value of adopting the strategy of comparing

normal with noninvasive tumors because it more clearly identifies genes involved in tumor initiation rather than progression, allowing a better understanding of the biology of the disease.

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