Identification of Genes Correlated with Early-Stage Bladder Cancer Progression

Randolph Stone II, Anita L. Sabichi, Jennifer Gill, I-ling Lee, Patrick Adegboyega, Michael S. Dai, Raja Loganantharaj, Marjan Trutschi, Urska Cvek, and John L. Clifford

Abstract

Transitional cell carcinoma (TCC) of the bladder ranks fourth in incidence of all cancers in the developed world, yet the mechanisms of its origin and progression remain poorly understood. There are also few useful diagnostic or prognostic biomarkers for this disease. We have combined a transgenic mouse model for invasive bladder cancer (UPII-SV40Tag mice) with DNA microarray technology to determine molecular mechanisms involved in early TCC development and to identify new biomarkers for detection, diagnosis, and prognosis of TCC. We have identified genes that are differentially expressed between the bladders of UPII-SV40Tag mice and their age-matched wild-type littermates at 3, 6, 20, and 30 weeks of age. These are ages that correspond to premalignant, carcinoma in situ, and early-stage and later stage invasive TCC, respectively. Our preliminary analysis of the microarray data sets has revealed ~1,900 unique genes differentially expressed (≥3-fold difference at one or more time points) between wild-type and UPII-SV40Tag urothelium during the time course of tumor development. Among these, there were a high proportion of cell cycle regulatory genes and a proliferation signaling genes that are more strongly expressed in the UPII-SV40Tag bladder urothelium. We show that several of the genes upregulated in UPII-SV40Tag urothelium, including RacGAP1, PCNA, and Hmmr, are expressed at high levels in superficial bladder TCC patient samples. These findings provide insight into the earliest events in the development of bladder TCC as well as identify several promising early-stage biomarkers.

Introduction

Transitional cell carcinoma (TCC) of the bladder causes substantial morbidity and mortality and has the fourth highest incidence of all cancers in the developed world, with an estimated 70,000 new cases and cases predicted to occur in the United States in 2009 (1). The number one correlate with bladder cancer is smoking. The majority of newly diagnosed cases (approximately two of three) are confined to the urothelium (do not breach the lamina propria) and are hence "superficial" or superficially invasive (2). There are a few commercially available urine-based tests for screening and surveillance for bladder cancer, but none of these can detect premalignancy (3, 4). Cytologic abnormalities of the urothelium are associated with carcinoma in situ (CIS), and urine cytology is positive in 90% of cases because of cell shedding into the urine due to the loss of cellular adhesiveness. However, cytology has a very low sensitivity and specificity for the detection of lower grade TCC. In addition, CIS is frequently associated with synchronous urothelial tumors of any stage. Strong indirect evidence indicates this lesion is a likely precursor of invasive carcinoma, but direct evidence in humans is lacking (5). Urothelial CIS has a high likelihood (>80%) of progressing to invasive carcinoma if left nontreated. Patients with TCC require frequent cystoscopic examination. If a tumor is found, treatment is transurethral resection and intravesical treatment. Cystectomy is required for invasive TCC confined to the bladder (6). Traditional prognostic factors (tumor stage and grade) do not sufficiently predict disease course or prognosis in the individual patient. Long-term study results clearly indicate that the ability to intervene at early stages and to monitor the success of treatment requires the definition of early markers for bladder cancer. It is therefore of vital importance to identify gene expression changes that occur during these early stages of bladder carcinogenesis and progression.

In an attempt to generate a mouse model for bladder cancer progression, investigators in the laboratory of Xue-Ru Wu have engineered transgenic mice carrying a low copy number of the SV40 large T (SV40T) oncogene, expressed...
under the control of the bladder urothelium–specific murine uroplakin II promoter (UPII-SV40Tag mice; ref. 7). The SV40T oncogene can bind and inactivate the p53 and Rb tumor suppressor genes (8), both of which are frequently mutated in human bladder TCC (9). In quiescent cells, Rb is bound to E2F family transcription factors, suppressing their ability to activate the transcription of genes required for DNA replication, nucleotide metabolism, DNA repair, and cell cycle progression (8, 10). SV40T blocks the Rb-mediated repression of E2F proteins, thereby inducing the expression of E2F-regulated proteins such as cyclins E, A and D1, chk1, fen1, BRCA1, and many others. UPII-SV40Tag mice develop a condition closely resembling human CIS starting as early as 6 weeks of age. This condition eventually progresses to invasive TCC from 6 months of age onward. Histologic examination of the bladder CIS lesions closely mimics the human histology (7).

There has been extensive effort in recent years aimed at identifying genetic markers, small biomolecules, and proteins as biomarkers for bladder cancer diagnosis, prediction of recurrence, as well as for surrogate endpoints in chemoprevention trials (3, 11). Ideally, such biomarkers should be detectable by noninvasive means, should be accurate and sensitive, and should provide a viable alternative to cystoscopy, which is invasive and can have a sensitivity as low as 70% (12). In spite of the identification of several promising markers by several groups, none have yet been able to meet these criteria (13–15). Therefore, the search for noninvasive biomarkers (i.e., in urine) demands a more guided approach. To this end, we have combined the UPII-SV40Tag mouse model for bladder cancer progression with Affymetrix microarray technology to determine the gene transcription profiles of urothelium from the UPII-SV40Tag mice and age-matched nontumorigenic littermates (wild-type, WT) at different times during the course of tumor development. We have identified ~1,900 unique genes differentially expressed (≥3-fold difference at one or more time points) between WT and UPII-SV40Tag urothelium during the time course of tumor development. Among these, there was a high proportion of cell cycle regulatory genes and proliferation signaling genes that were more strongly expressed in the UPII-SV40Tag bladder urothelium.

Materials and Methods

UPII-SV40Tag transgenic mice

UPII-SV40Tag mice were previously generated in the laboratory of Xue-Ru Wu (NYU School of Medicine, Kaplan Comprehensive Cancer Center, Department of Urology, New York, NY) on an FVB/N background (7) and were a generous gift. All mice referred to as UPII-SV40Tag were hemizygous for transgene expression. WT control mice were age matched from the same litters as the transgenics. Genotypes were determined by PCR from tail genomic DNA preps using the following primers for the SV40Tag: 5′-TTCATGCCCCTGAGTCTTCCAT-3′ and 5′-GCCAGGAAAATGCTGATAAAAATG-3′.

Small animal magnetic resonance imaging

Mice were imaged using a Signa 1.5 T MR scanner. The mice were anesthetized using isoflurane inhalation anesthesia, and contrast was injected through the tail vein. To validate the magnetic resonance imaging (MRI) method of in vivo tumor detection in a mouse model, we scanned and subsequently sacrificed mice at 3, 4, 6, 8, and 12 months of age, and compared image and histopathologic assessment of the genitourinary tract. T2-weighted and T1-weighted plus contrast images were obtained for each mouse.

RNA isolation

Normal urothelium, hyperplastic/CIS urothelium, or TCC tissue was dissected from the bladder wall and snap frozen. Total cell RNA was isolated by homogenization of frozen tissue using TriReagent (Molecular Research), followed by standard organic extraction and precipitation and purification on RNaseasy RNA purification columns (Qiagen). Purity and yield were determined by UV absorbance over the range of 220 to 320 nm. Total RNA was extracted from each human bladder–derived cell line using Trizol (Molecular Research Center) according to the manufacturer’s instructions. RNA concentrations were determined using a SmartSpec Plus Spectrophotometer (Bio-Rad).

DNA microarrays

All DNA microarray procedures were done in the Louisiana State University Health Sciences Center-Shreveport DNA Array Research Core Facility. Probe synthesis and array hybridization were done using established methods provided by Affymetrix. Briefly, 2 μg of purified urothelial cell RNA were reverse transcribed into cDNA, using a T7 promoter-(dT)24 primer. Following second-strand synthesis, biotin-labeled cRNA was generated from the double-stranded template using T7 RNA polymerase. The quality of the cRNA probe was verified by running an aliquot on an agarose gel. Exactly 20 μg of the labeled cRNA were hybridized to the Affymetrix GeneChip Mouse Expression Set 430 chip for 16 hours at 45 °C in 300 mL of premixed hybridization solution containing labeled hybridization control prokaryotic genes (bioB, bioC, bioD, and cre). Replicate spots for each control gene are present on the chip. Chips were washed in the GeneChip Fluidics Station automatic washer and scanned on the GeneArray fluorometric scanner. The data files were then transferred to one of the DNA Array Research Core computers for analysis.

Biometric analysis of microarray data

Identification of gene expression differences between microarrays was conducted within the DNA Array Core by Drs. Marjan Trutschl and Urska Cvek (Department of Computer Science and Mathematics, Louisiana State University-Shreveport). The Affymetrix GeneChip Operating Software
(GCOS, Affymetrix, Inc.) and the R project (16) were used to analyze the data, combined with proprietary visualization software developed at LSU-S Laboratory for Advanced Biomedical Informatics. The total number of chips analyzed for this project was as follows: four time points (3, 6, 20, and 30 wk) × two mouse types (and UPII-SV40Tag and nontransgenic littermates) × two replicate bladder urothelium RNA preps (except for the 6-wk nontransgenic sample, which only had a single sample) for a total of 15 chips. We identified the genes expressed in the urothelium of UPII-SV40Tag and nontransgenic littermates at the four time points. Signal values and fold changes were determined by GCOS. Heat map visualization was generated by using the WT to UPII-SV40Tag fold changes at each of the time points (3, 6, 20, and 30). The difference is marked as upregulated (red), if the fold change between the UPII-SV40Tag and WT was >1, and downregulated (green), if it was smaller than 1. We used the Ingenuity Pathways Analysis software package (Ingenuity Systems, Inc.) to explore signaling pathway and cell function ontologies between groups.

**Semiquantitative reverse transcriptase-PCR**

Semiquantitative reverse transcriptase-PCR (RT-PCR) was done essentially as previously described (17, 18). RNA (∼2 μg/reaction) was used to generate cDNA, and the appropriate individual pairs of 20mer to 30mer oligonucleotides (50 pmol/reaction) for the test genes (Table 1) were used to amplify DNA from the cDNA. Semiquantitative PCR was done by using 100 μL reaction volumes and taking 33 μL aliquots at 25, 30, and 35 cycles. The expression of mRNA for the cytoskeletal protein β-actin or 36B4, the gene for the ribosomal phosphoprotein P0, which are both ubiquitously expressed, was determined for each RNA sample to control for variations in RNA quantity. Ten microliters of each reaction was electrophoresed in a 2% agarose gel containing ethidium bromide. The gel was then developed using the GelDoc XR system (Bio-Rad) and quantified using Quantity One (Bio-Rad).

**Cell lines**

UM-UC-10 and UM-UC-13 cells were cultured essentially as previously described in ref. (19). Cells were cultured in 50% DMEM low-glucose/50% F12 medium (DMEM/F12) containing 10% fetal bovine serum. SV-HUC cells were cultured in F12+ media (F12 supplemented with 2.7 g/L dextrose, 0.1 mmol/L nonessential amino acids, 0.2 mmol/L L-glutamine, 200 U/L insulin, 1 mg/mL human transferrin, and 1 mg/mL hydrocortisone) containing 5% FCS at 37°C in the humidified atmosphere of 5% CO₂/95% air. Primary human urothelial cells (ScienCell Research Laboratories) were cultured according to the manufacturer's protocol. Primary Human urothelial cells were cultured in urothelial cell medium supplemented with 1% urothelial cell growth supplement (UCGS) and 1% penicillin/streptomycin solution (P/S). All cells were cultured at 37°C in the humidified atmosphere of 5% CO₂/95% air.

### Table 1. Primers used

<table>
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**RT-PCR primers for testing RNA from WT and UPII-SV40Tag mice**

RT-PCR primers for testing RNA from WT and UPII-SV40Tag mice were used to amplify DNA from the cDNA. Semiquantitative PCR was done by using 100 μL reaction volumes and taking 33 μL aliquots at 25, 30, and 35 cycles. The expression of mRNA for the cytoskeletal protein β-actin or 36B4, the gene for the ribosomal phosphoprotein P0, which are both ubiquitously expressed, was determined for each RNA sample to control for variations in RNA quantity. Ten microliters of each reaction was electrophoresed in a 2% agarose gel containing ethidium bromide. The gel was then developed using the GelDoc XR system (Bio-Rad) and quantified using Quantity One (Bio-Rad).

**Immunohistochemical examination of human superficial bladder cancer specimens**

Tissue obtained from transurethral resection was fixed in formalin and embedded in paraffin before sectioning. Paraffin sections of 4 μm were deparaffinized in xylene for 3 × 7 minutes at room temperature and rehydrated by stepwise washes in decreasing ethanol/H₂O ratio (100-50%, followed by soaking in water). Sections were incubated in the Superblock (Pierce Biotechnology, Inc.) blocking reagent for 1 hour at room temperature to block nonspecific antigen sites. After washing thrice in PBS, slides were incubated overnight at 4°C with antibodies against RacGAP1, PON3, cytokeratin 19 (K19), and II18 (Abcam, Inc.); survivin (Birc5)
and proliferating cell nuclear antigen (PCNA; Cell Signaling); and RHAMM (Santa Cruz). Colorimetric detection was done according to the instructions for the Vectastain Elite ABC kit (Vector Laboratories). Fluorimetric detection was done using Alexa-488–labeled secondary antibodies, and sections were counterstained with 4′,6-diamidino-2-phenylindole. Slides were photographed on a Nikon TE300 microscope either with bright-field or fluorescence conditions, with a charge-coupled device camera (Roper Scientific). Images were processed with the IPLabs v3.55 software (Scanalytics, Inc.).

Results

The UPII-SV40Tag model recapitulates invasive bladder TCC

In this study, we have used the UPII-SV40Tag mouse model of bladder cancer progression, in combination with comprehensive DNA microarray analyses, to explore early events in the development of bladder cancer and to identify potential biomarkers for bladder premalignancy. An initial goal was to better characterize the earliest macroscopic changes in bladder tissue in live UPII-SV40Tag mice using small-animal MRI techniques developed at the M.D. Anderson Cancer Center's experimental animal imaging facility. We scanned and subsequently sacrificed mice at ages ranging from 6 weeks to 12 months of age and compared MR image and histopathologic assessment of the genitourinary tract. An example of axial T1 postcontrast and T2 MR images of the urinary bladder of a 6-week-old mouse that showed moderate irregular thickening of the urinary bladder mucosa, which corresponded to histologic findings of diffuse hyperplasia of the transitional epithelium is shown in Fig. 1 (Fig. 1D-F). At 38 weeks of age, MRI identified large, irregular contrast-enhancing masses...
within the bladder lumen, some of which had invaded into the surrounding abdominal cavity (Fig. 1H and I, arrows). These tumors were carcinoid in appearance and without the delicate papillae that characterize papillary tumors. We found a close correlation between MR image and histologic detection of intravesical abnormalities in the mice in all age groups (Fig. 1, compare A, D, and G to other panels).

**Gene expression profile of bladder cancer progression**

In parallel with the histologic and macroscopic characterization, we used the Affymetrix DNA microarray technology to compare the gene transcription profiles of normal bladder urothelium (from nontransgenic littermates) with the urothelium of the UPII-SV40Tag mice, over time. We chose to examine mice at 3, 6, 20, and 30 weeks of age. These times for comparison encompass early-stage changes that precede the appearance of CIS (3 wk), CIS (6 wk), and early-stage and later stage TCC (20 and 30 wk, respectively). Our determination of genes expressed in the urothelium at these time points revealed ~1,900 unique differentially expressed (≥3-fold difference) genes at one or more of the time points between the urothelium of UPII-SV40Tag mice and their age-matched WT littermates (see Supplementary Table S1 for the full list). Figure 2 illustrates the clustering of the differentially expressed genes according their expression patterns over the time course. Genes more highly expressed in the UPII-SV40Tag bladders are shown in red, and genes more strongly expressed in WT bladders are shown in green. Black bars indicate genes that are expressed at similar levels in both mouse lines for that time point. We focused attention on a group of genes with a high fold increase in expression in UPII-SV40Tag mice for all four time points (Fig. 2, expanded left). We reason that this group of genes could contain candidate biomarkers for both premalignant and later stage TCC. The time course of expression of some of the most strongly upregulated and downregulated genes are shown graphically (Fig. 3A, higher expression in UPII-SV40Tag; Fig. 3B, lower expression in UPII-SV40Tag). Interestingly, some genes, such as BRCA1, were strongly expressed in premalignant urothelium only at the early stages of progression, with levels eventually dropping to near normal by later stages. We note a high proportion of genes involved in cell proliferation among the upregulated genes, and several structural and differentiation-related genes were among the downregulated genes. The microarray results were confirmed independently by RT-PCR for several of the genes (Fig. 4). For all genes tested to date, the relative direction of expression between WT and UPII-SV40Tag bladders was the same for both the RT-PCR and microarray results.

**Gene network and pathway analysis**

We next performed biometric analysis using the Path Explorer function in the Ingenuity Pathways Analysis software package (Ingenuity Systems, Inc.) on the list of the 1,900 differentially expressed genes (≥3-fold up or down at 1 or more time points), considering the expression differences between WT and UPII-SV40Tag mice separately for each time point. There was an average of 45 biological networks generated for the gene lists for each time point. Biological networks are defined as highly connected networks of up to 35 genes. A significance score based on a P value calculation is assigned to each network and is displayed as a negative log of the P value. The higher the score, the less likely it is that the set of genes from our list appearing in the network (focus genes) could be explained by random chance alone. See Supplementary Table S2 for a full list of networks, their significance scores, number of focus molecules, relative direction of expression, and their top functions. The 30 top-scoring gene networks were identical for all time points, although not all gene changes were the same in each network for each time point. This similarity between time points is expected because the same 1,900-gene list was used for each analysis, with only the fold difference between WT and UPII-SV40Tag varying between sets. The similarity indicates that most of the major gene expression changes start to take place as early as 3 weeks. It should be noted that the 3-week time point precedes the appearance of invasive TCC by several weeks, such that gene expression differences at this time could be considered representative of a premalignant state. The top-scoring networks contain genes involved in the cell cycle; DNA replication, recombination, and repair; cancer; cellular movement; and cellular assembly and organization. The merged image of the top three networks for the 3-week time point indicates three nodes centered on JUN, extracellular signal-regulated kinase (ERK), and P21, all key regulators of proliferative responses (Fig. 5). Some of the other genes that are upregulated in UPII-SV40Tag mice include those encoding centromere proteins Cenpa, Cenpf, and Cenph; Aurora kinases A and B; cyclins ccnb1, ccnb2, ccne2, cnna2, and cnnf; cell division cycle proteins Cdc7, Cdc2a, Cdc20, Cdc6, and Cdc3; kinesin-like family proteins, kif1c, kif2c, kif11, kif20a, kif 22, and kif23; multiple minichromosome maintenance–deficient proteins MCM2, MCM4, MCM5, MCM6, MCM7,8, and MCM10; other proliferation-related proteins such as E2f8, Spbc24, Top2a, Brca1, RacGAP1, Hmmr, and others (Supplementary Table S1; Figs. 2 and 5). Many of these genes are common with the SV40T/t-antigen cancer signature identified recently by Deeb et al. (20) for human breast, prostate, and lung carcinomas. In addition, we have identified several genes that are suppressed in the UPII-SV40Tag bladders relative to WT littermates, which includes a large proportion of structural and cell adhesion genes that seem to be related to the normal differentiated state of urothelium. Examples are genes encoding extracellular matrix proteins such as collagens Col1a1, Col1a2, Col6a2, Col3a1, laminin B1, and tenascin C; keratins knr2-5, knr1-15 and other intermediate filament proteins Dnm, and Vim; as well as uroplakins upk1b and upk2 itself, and other tight junction proteins cldn8, ctnnb1, ctnnal1, ctnnd2, pcdhgc3, and cgnl1. Other downregulated genes that are potentially involved in the development of TCC include superoxide dismutase 3 (SOD3), cyclin D2...
Fig. 2. Preliminary hierarchical clustering of all four time points at 3, 6, 20, and 30 wk. The colors red, green, and black represent genes that are upregulated, downregulated, or no change, respectively, in the UPII-SV40Tag mice when compared with the WT littermates. The last cluster, cluster 8, has been enlarged and rescaled, and represents genes that are highly upregulated at all four time points.
ccnd2), transthyretin (Ttr), bone morphogenetic protein 2 (BMP2), and matrix metalloproteinase 2 (Mmp2). As in Fig. 1, red and green indicates higher expression in the UPII-SV40Tag and WT bladders, respectively.

Differentially expressed genes in human bladder-derived cell lines and in human superficial bladder cancer

Finally, we have attempted to determine the relevance of several of the differentially expressed genes to human bladder cancer. We first compared mRNA expression levels for several genes in human normal urothelial cells (primary HUCs), “premalignant” urothelial cells (SV-HUC), and advanced TCC cells (UM-UC-10 and UM-UC-13). We have recently described the UM-UC cells in detail (21). The UM-UC-10 cells were derived from a bladder tumor, have mutant p53, undetectable levels of Rb, and are nontumorigenic in nude mice. The UM-UC13 cells were derived from a lymphatic metastasis, also have mutant p53 and undetectable RB, but are tumorigenic in nude mice. We observed that more than half of the genes tested by semiquantitative RT-PCR were expressed as predicted in the human cell lines, such that genes overexpressed in the UPII-SV40Tag mice were more strongly expressed in the premalignant

Fig. 3. A, selected genes that were expressed at higher levels in UPII-SV40Tag urothelium relative to WT littermates are shown. Black columns, raw expression values for UPII-SV40Tag mice (n = 2). White columns, expression values for WT mice. B, selected genes that were expressed at lower levels in UPII-SV40Tag urothelium relative to WT littermates.
and malignant cell lines (data not shown; Fig. 6A). Conversely, Ccnd2, which was downregulated in the UPII-SV40Tag mice, is expressed only in the primary HUCs.

Next, we tested the expression of several differentially expressed genes in paraffin sections of high- and low-grade superficial bladder TCC samples that were excised by transurethral resection. We prioritized biomarkers for initial testing based on whether the genes that were found to be highly expressed at all four time points (PCNA, Survivin, Hmnr, and RacGAP1) as shown in Fig. 2 (left) are cell surface proteins (PON3) or are secreted (IL18). We reason that such proteins would also have a higher likelihood of
being detectable in urine. To date, we have tested the expression of six proteins in tumor samples from 12 patients (6 high grade and 6 low grade). All six of these proteins were detectable by immunohistochemical staining in the patient samples, with the strongest expression detected for RacGAP1, PCNA, and Hmmr (data not shown; Fig. 6B). This expression also colocalized with the expression of cytokeratin 19 (K19), a urothelial marker. Hmmr is expressed evenly throughout the cytoplasm, whereas PCNA is strongly expressed in the nuclei of hyperplastic urothelial cells, as previously described by others (Fig. 6B, lower row; refs. 22, 23).

We note that RacGAP1 is expressed in the cytoplasm, with prominent focal perinuclear staining, which agrees with our own immunocytochemical staining of bladder TCC cell lines (data not shown). It is not yet possible to determine whether there is a statistically significant difference in expression for any of the markers between high- and low-grade TCC due to the low sample size.

**Discussion**

These findings represent what is to our knowledge the first attempt to obtain a comprehensive gene expression profile of bladder premalignancy. This is part of our
long-term effort to identify biomarkers of the earliest stage bladder TCC, which could potentially predict occurrence and recurrence of bladder TCC. A more immediate aim of these studies is to identify potential biomarkers of early-stage bladder TCC that can be tested in patient urine, bladder wash, and other tissue samples. We have identified ~1,900 genes that are differentially expressed (>3-fold higher or lower) between the bladder urothelium of UPII-SV40Tag mice and their age-matched WT littermates at ages that encompass early-stage changes that precede the appearance of CIS (3 wk), CIS (6 wk), and early-stage and later stage TCC (20 and 30 wk, respectively).

A large proportion of the genes upregulated in the UPII-SV40Tag urothelium are cell cycle–regulatory and proliferating-signaling genes. Many of these genes are common with the SV40T/t-antigen cancer signature identified recently by the laboratory of J.E. Green and collaborators (20). These investigators used DNA microarrays to compare three transgenic mouse models for breast, lung, and prostate cancer, all based on tissue-specific expression of SV40Tag, and found a common set of differentially expressed genes that are involved in cell proliferation, DNA repair, and apoptosis. Of the 119 genes that comprise this T/t-antigen signature, 73 are found in our list of ~1,900 differentially expressed genes (61% identity), suggesting similarity between models. Most importantly, this same signature of genes was associated with the most aggressive tumor phenotype and poor prognosis in human breast, lung, and prostate cancer (20). Whether the same association exists with human bladder TCC remains to be determined. In addition to overexpressed genes, we have identified several genes that are suppressed in the UPII-SV40Tag bladders relative to WT littermates. This includes structural and cell adhesion genes that seem to be related to the normal differentiated state of urothelium. These include genes encoding extracellular matrix proteins, intermediate filament proteins, as well as uroplakins and other tight junction proteins. Supplementary Table S1 contains the full list of genes that were found to be differentially expressed (≥3-fold at one or more time points) between UPII-SV40Tag and WT littermate urothelium.

We used the Ingenuity Pathways Analysis software package to analyze the microarray data sets to identify the predominant cellular functions and signaling pathways that distinguish the earliest accessible stage of bladder TCC in the UPII-SV40Tag model. When we examined the biological networks that are derived from the 1,900 differentially expressed gene list, we noted that the top-scoring networks contain genes involved in the cell cycle; DNA replication, recombination, and repair; cancer; cellular movement; and cellular assembly and organization. The three highest scoring networks center on the activator protein 1 (AP-1) transcription factor subunit, JUN, the mitogen-activated protein kinase ERK, ERK, and the cyclin-dependent kinase inhibitor, P21 (Fig. 5). These are regulators of proliferative responses and are part of linked pathways all known to be affected by oncogenic mutations (24). AP-1 is a positive regulator of cell proliferation and transformation, and its activity is stimulated in mouse skin tumorigenesis models by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (25). A direct link between between mitogen-activated protein signaling and AP-1 activity has been established from studies in which kinase-deficient forms of ERK could inhibit AP-1 activation by several stimuli (26, 27). Those findings have led to several studies, including our own, aimed at understanding the mechanism of suppression of mitogen-activated protein kinase signaling and/or AP-1 activity by the vitamin A metabolite all-trans retinoic acid, an efficient suppressor of tumor formation in several epithelial cancer models (28–30). Our recent studies have shown that the B-Raf/Mek/Erk mitogen-activated protein kinase pathway is a target for the chemopreventive activity of all-trans retinoic acid in the mouse two-stage skin carcinogenesis model (29). The present gene network analysis suggests that this same pathway could also be a target for the prevention of bladder TCC and will guide future experiments for testing potential chemopreventive and/or therapeutic drugs such as all-trans retinoic acid.

In an effort to narrow our candidate list of biomarkers to those most likely to be involved in the earliest stages of TCC, we have focused on genes differentially expressed at the 3-week time point. Among these genes, we have further focused on those which also remain highly differentially expressed at later time points, with the expectation that such genes could also serve as markers for later stage TCC (Fig. 2, left). In addition, to increase the likelihood of detection by antibodies in urine samples, we are paying closest attention to proteins that are secreted or known to reside in the plasma membrane and, with few exceptions, those which have been previously detected in urine or bladder tissue by other investigators. These include hyaluronan-mediated motility receptor (Hmmr/Rhamm), which has been identified as highly expressed in early stage (Ta and T1), and later stage (T2-4) bladder TCC (22), PCNA (23), autocrine motility factor receptor (31) and others. We have tested for the expression of several candidate genes that are upregulated in UPII-SV40Tag mice at all four time points (RacC1, PCNA, Survivin, and Hmmr), are upregulated and secreted (IL18), and are upregulated and expressed at the cell surface (PON3), in paraffin sections of high- and low-grade superficial bladder TCC samples. We detect all of these proteins to varying degrees in the tumor samples, with the highest levels detectable for RacC1, PCNA, and Hmmr (Fig. 6B). These data, although preliminary, provide strong support for further testing and validation of these genes as biomarkers for superficial (early stage) TCC. We have also begun testing for the expression of these proteins in urine samples from a recently completed phase II, randomized, placebo-controlled chemoprevention trial (N01 CN55186, PI: A. Sabichi) that was designed to test whether celecoxib can prevent recurrence in patients successfully treated by transurethral resection for nonmuscle invasive bladder cancer. In this trial, urine samples were collected over the course of treatment every 3 months for 18 months after curative therapy (transurethral resection plus Bacillus Calmette-Guerin), or until the time of recurrence (~30% of
patients). It is anticipated that single markers or combinations of markers can be validated for the prediction of recurrence and possibly for the prediction of response to therapy. In the future, we will also focus attention on proteins predicted to be downregulated in premalignant urothelium, such as uroplakin II, bone morphogenetic protein 2, and superoxide dismutase 3. These could serve as negative markers for recurrence.

We have identified genes that are differentially expressed in premalignant urothelium, in a mouse model for aggressive bladder TCC. This group of genes now serves as a promising pool of candidates for biomarkers for early-stage TCC, as well as a source for gaining insight into the earliest events preceding early-stage TCC and/or CIS. Future experiments are aimed at validating promising biomarkers in larger numbers of patient tumor samples and in urine, as well as exploring the molecular roles of individual differentially regulated genes (e.g., Hmmr) in bladder premalignancy and early-stage bladder TCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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