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Gene Expression Profiling of Chemically Induced Rat Bladder Tumors

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Abstract

A variety of genetic alterations and gene expression changes are involved in the pathogenesis of bladder tumors. To explore expression changes in 4-hydroxybutyl(butyl)nitrosamine–induced rat bladder tumors, microarray analysis was performed. Analysis yielded 1,138 known genes and 867 expressed sequence tags that were changed when comparing tumors to normal rat epithelia. Altered genes included cell cycle–related genes, EGFR-Ras signaling genes, apoptosis genes, growth factors, and oncogenes. Using the pathway visualization tool GenMAPP, we found that these genes can be grouped along several pathways that control apoptosis, cell cycle, and integrin-mediated cell adhesion. When comparing current data with previous mouse bladder tumor data, we found that > 280 of the same known genes were differentially expressed in both mouse and rat bladder tumors, including cell cycle–related genes, small G proteins, apoptosis genes, oncogenes, tumor-suppressor genes, and growth factors. These results suggest that multiple pathways are involved in rat bladder tumorigenesis, and a common molecular mechanism was found in both rat and mouse bladder tumors.

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Introduction

Bladder cancer is the fifth most common cancer in the United States and is associated with exposure to cigarette smoke. Approximately 15% of bladder tumors evolve into invasive tumors after infiltration through the basement membrane. Patients with muscle-invasive disease are at high risk for recurrence, progression, and metastasis. Although early-stage bladder cancer can be treated surgically, the rate of recurrence is quite high [1]. Significant progress has been made in understanding the underlying molecular and genetic events in bladder cancer. Numerous markers have been described to correlate, to some extent, tumor stage and the prognosis of patients with bladder cancer [2]. Although a number of markers have been identified, there remains a need for the development of reliable additional markers that can provide information regarding diagnosis and prognosis. In addition, the identification of specific proteins that might be favorable targets for treatment is of some interest. Expression profiling with high-throughput DNA microarrays has the potential of providing critical clues. Our previous study on mouse bladder tumors revealed that activation of the EGFR-Ras, G13, and TGF-β pathways, and increased cell proliferation appear to play important roles during mouse bladder tumorigenesis.

There are two primary chemically induced models of urinary bladder cancers in rodents. Both employ repeated intragastric administration of 4-hydroxybutyl(butyl)nitrosamine (OH-BBN) to induce bladder cancers in either mice or rats [3,4]. Bladder cancers typically have a mixed histology, showing elements of both transitional and squamous cells. Investigators have found a relatively low frequency of *ras* mutation in these cancers [5]. However, roughly 50% of these tumors develop *p53* mutations [6]—a percentage similar to that found in humans. There has been further characterization of these tumors for various gene products, including mutations in the epidermal growth factor receptor (*EGFR*) kinase activation loop [7]. Similar to human bladder tumors, these tumors tend to show overexpression of *EGFR* and amphiregulin. Other genetic changes include *ras*, *erb-B2*, and *EGFR*. The transforming potential of *ras* is due to mutation, whereas *EGFR* and *erb-B2* are overexpressed in transformed cells. Reported frequencies of H-*ras* point mutations with a glycine-to-valine substitution in codon 12 in bladder neoplasms vary widely from 0% to 45% between studies [8–11]. Recently, several means of suppressing *ras* activity, including inhibitors of *ras* signal transduction and a *ras*-suppressor mutant, have been reported [12]. Overexpression of *EGFR* or *erb-B2* and *ras* mutations results in constitutive

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MAPK activation [13], and this correlates with muscular invasion and extent of tumor invasion [2]. Almost all advanced bladder carcinomas exhibit alterations in cell cycle genes (e.g., decreases in *pRb* or $p16^{INK4a}$, or increases in *cyclin D1* expression preferentially occurring in earlier stages) [14,15].

In this study, we employed Affymetrix GeneChips (Affymetrix, Santa Clara, CA) representing > 30,000 genes and expressed sequence tags (ESTs) to identify differentially expressed genes in rat bladder tumors. The objectives of the study were: 1) to detect and identify differential gene expression profiles in rat bladder tumors; 2) to help elucidate the underlying mechanisms of rat bladder tumorigenesis; and 3) to compare the present results with our previous data on mouse bladder tumors to identify common genes and pathways that may be particularly relevant to the mechanism of carcinogenesis in the bladder.

Materials and Methods

Rat Bladder Tumors

Rats were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN), at 28 days of age and were housed in polycarbonate cages (five per cage). The animals were kept in a lighted room 12 hours each day and maintained at 22 ± 0.5°C. Teklad 4% mash diet (Harlan Teklad, Madison, WI) and tap water were provided *ad libitum*. At 56 days of age, mice received the first of 12 weekly gavage treatments with OH-BBN (TCI America, Portland, OR). Each 7.5-mg dose was dissolved in 0.1 ml of ethanol/water (25:75). Rats (unless sacrificed early because of a large palpable bladder mass) were sacrificed 8 months following the first OH-BBN treatment. Bladder tumors were removed and frozen for subsequent molecular assays. A portion of each tumor was fixed and processed for routine paraffin embedding, cut into 5-μm sections, and mounted for hematoxylin–eosin staining for histopathology. All bladder tumors used in this study were diagnosed as bladder carcinomas, with a mixed histology showing elements of both transitional and squamous cells. Both bladder tissues and normal bladder epithelia came from age-matched controls.

RNA Isolation and Amplification

To isolate bladder epithelia, we separated the epithelia from the stroma and muscle tissues by cutting the bladder into half and scraping off the epithelium. Total RNA from normal bladder epithelia and bladder tumors were isolated by Trizol (Invitrogen, Carlsbad, CA) and purified using the RNeasy Mini Kit and RNase-free DNase Set (QIAGEN, Valencia, CA) according to the manufacturer's protocols. *In vitro* transcription-based RNA amplification was then performed on each sample. cDNA for each sample was synthesized using a Superscript cDNA Synthesis Kit (Invitrogen) and a T7-(dT)24 primer, 5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24-3′. cDNA were cleaned using phase-lock gels (Fisher cat ID E0032005101) and phenol/chloroform extraction. Then, biotin-labeled cRNA were transcribed *in vitro* from cDNA using a BioArray High Yield RNA Transcript Labeling Kit (ENZO Biochem, New York, NY) and purified again using the RNeasy Mini Kit.

Affymetrix GeneChip Probe Array and Quantitative Confirmation by Real-Time Polymerase Chain Reaction (PCR)

The labeled cRNA were applied to Affymetrix Rat 230 2.0 GeneChips (Affymetrix) according to the manufacturer's recommendations. Every gene or EST is represented by a probe set consisting of approximately 16 probe pairs (oligonucleotides) of 25-mer oligonucleotides. One sequence of a probe pair represents the complementary strand of the target sequence, whereas the other has a 1-bp mismatch at the central basepair position. This mismatch sequence serves as an internal control for the specificity of hybridization. To evaluate the reliability of array results, genes were randomly selected from the genes detected in the microarray assay for further confirmation by real-time PCR, as previously described [16]. The large number of differentially expressed genes led us to take a further quality control step in which the distribution of fold changes was examined.

Cluster and GenMAPP

Array normalization and gene expression estimates were obtained using Affymetrix Microarray Suite 5.0 software (MAS5). Array mean intensities were scaled to 1500. These estimates formed the basis for statistical testing. Differential expression was determined using the combined basis of *t*-test with $P < .05$ and fold changes (either up or down) of > 2-fold. Genes meeting both criteria were called positive for differential expression. Hierarchical clustering was then performed as follows. For selected genes, expression indexes were transformed across samples to an $N(0,1)$ distribution using a standard statistical *Z*-transform. These values were put into the GeneCluster program of Eisen et al. [17], and genes were clustered using average linkage and correlation dissimilarity. Signal transduction pathways, metabolic pathways, and other functional groupings of genes were evaluated for differential regulation using the visualization tool GenMAPP [18]. We imported the statistical results of our data set into the program and used GenMAPP to illustrate pathways containing differentially expressed genes.

Protein Isolation and Two-Dimensional (2D) Protein Gel

Protein was isolated from epithelia and tumors, and proteomic analysis was performed using 2D differential gel electrophoresis. Protein samples from five normal and five tumor-bearing animals were paired. The protein samples (50 μg) were labeled substoichiometrically with one of two *N*-hydroxysuccinimide cyanine dyes (GE Healthcare Biosciences, Piscataway, NJ). Immobilized pH gradient strips (24 cm; pH 3–11, nonlinear) were rehydrated with the samples (pooled normal and tumor). The first dimension of isoelectric focusing was performed for 75 kV h in Protean IEF Cell System (Bio-Rad, Hercules, CA). The strips were equilibrated and positioned on a 10% to 20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. Resolved protein images were acquired on a Typhoon

9400 scanner (GE Healthcare Biosciences). Relative quantification of matched gel features was performed using Decyder-DIA software (GE Healthcare Biosciences). Selected gel features were excised and digested *in situ* with trypsin. The resulting peptide pools were analyzed by tandem mass spectrometry using both matrix assisted layer desorption/ionization tandem time-of-flight mass spectrometer (MALDI-TOF/TOF) (Proteomics 4700; Applied Biosystems, Framingham, MA, and Toronto, Ontario, Canada) and liquid chromatography–tandem mass spectrometer (LC–MS/MS) (LTQ-FTMS; Thermolelectron, San Jose, CA) instruments. Peptide fragmentation spectra were processed using Data Explorer v. 4.5 and Analyst software (Applied Biosystems, Foster City, CA), and MASCOT v. 1.9 (Matrix Sciences, London, UK).

Immunohistochemistry (IHC) Staining

Briefly, paraffin sections of rat normal and tumor bladders (n = 5) were antigen-retrieved in citrate buffer for 20 minutes in a microwave. This was followed by blocking in normal horse serum, and sections were incubated in primary antibody cyclin D1 (sc-450, 1:500; Santa Cruz Biotech, Santa Cruz, CA) or annexin I (sc-12740, 1:50; Santa Cruz Biotech) overnight at 4°C. The corresponding biotinylated secondary IgG (1:500) was used, and the sections were developed by the ABC method (Vector Laboratories, Burlingame, CA) with 3,3′-diaminobenzidine HCl as substrate.

Results

Gene Expression Profile in Bladder Tumors

Microarray data were compared for five rat bladder tumors and their age-matched normal rat bladder epithelia. The fold changes of gene expression were based on the ratios of mean values between tumors and epithelium controls. Two thousand five known genes and ESTs were found to be differentially expressed in rat bladder tumors with a fold change of ≥ 2 and $P < .05$. Among them, 1,138 genes were known genes, with 770 genes overexpressed and 368 genes underexpressed in bladder tumors (Figure 1). Many of the overexpressed genes were cancer-related genes belonging to EGFR-Ras signaling, cell cycle, and apoptosis (Table 1). Underexpressed genes included the Rab subfamily of genes, tumor-suppressor genes, and genes encoding casein kinases, cytochrome *P*450s, and RAR-related orphan receptors (Table 2). These genes are involved in a broad range of different pathways, including control of cell proliferation, differentiation, cell cycle, signal transduction, and apoptosis. Tables 1 and 2 list selected genes that were changed in rat bladder tumors. The Ras superfamily is a diverse group of small G proteins participating in many cellular processes and widely involved in tumorigenesis. In this study, many Ras superfamily members were found to be abnormally expressed in bladder tumors. Interestingly, Rab subfamily genes were underexpressed (Table 2). All other Ras-related genes, such as *Ras*, *Rin*, *Rem*, *Rap*, *Rac*, *Rad*, and *Rho*, were overexpressed in bladder tumors (Table 1). Many overexpressed genes in tumors were cell cycle–related genes that promote entry into cell cycle and mitosis. These include the following: *Cdc2A*, *Cdc20*, and *Cdc25B*; *cyclins A2*, *B1*, *B2*, and *D1*; *Cdkn2a*, *Cdkn2c*, *Cdkn2d*, and *Cdkn3*; *MAD2*; *Polo-like kinases 1* and *4*; and *Gadd45* β and γ (Table 1). A more limited number of cell cycle–related genes (e.g., *cyclin M1*, *p57*, and *JunDP1*) were underexpressed in rat bladder tumors (Table 2). Interestingly, these genes play important roles in cell cycle arrest and G_1/S and G_2 checkpoints. The *Kit*, *Maf*, *Fes*, *Myc*, and *Fyn* oncogenes were overexpressed in rat bladder tumors (Table 1), whereas the *WT1*, *BRCA2*, *Mycl1*, and *Pak* genes were underexpressed in rat bladder tumors (Table 2). *Survivin*, *TNF*, and *Bcl-2* were also overexpressed in rat bladder tumors (Table 1).

Confirmation of Differentially Expressed Genes for Both RNA and Protein Levels

With such a large number of differentially expressed genes, we examined the distribution of fold changes to detect if any large skew could account for the results. The distribution of fold changes for differentially expressed genes is shown in Figure 2*A*, and its symmetry suggests that no skew artifact is present. We validated the differential expression of 15 genes by real-time PCR. Thirteen of 15 genes were confirmed by real-time PCR. The confirmation rate is > 86% at a cutoff of two-fold change and ($P < .05$). The real-time PCR results of these 13 genes agreed well with microarray data (Figure 2*B*). We also examined several genes at protein level by 2D protein gel electrophoresis and IHC. The genes *annexin A1* and *cyclin D1* were chosen for this analysis, and results are presented in Figure 3. Both RNA and protein level changes for these genes agreed well with initial microarray changes.

Differentially Expressed Genes Interpreted by GenMAPP

We introduced the differentially expressed genes found in microarrays into GenMAPP. GenMAPP search revealed that apoptosis, cell cycle, and integrin-mediated cell adhesion are actively involved in bladder tumorigenesis, with expression changes in multiple genes, each of which may play important roles in the pathogenesis of bladder cancers. Figure 4 represents the genes differentially expressed in rat bladder tumors involved in these signaling pathways.

Comparison of Differentially Expressed Genes Between Rat and Mouse Bladder Tumors

We compared the differentially expressed genes from both rat and mouse bladder tumors using gene expression data from mouse bladder tumors [19]. Of 860 comparable genes between rats and mice, there were 280 genes that had the same tendency of expression changes in both mouse and rat bladder tumors. Selected genes were listed in Tables 1 and 2 and marked with asterisks. The majority of these genes are cell cycle–related genes, *ras* small G proteins, and apoptosis-related and growth factor–related genes.

Discussion

In this study, we have shown that microarray can be used to enhance the search for the molecular pathogenesis of

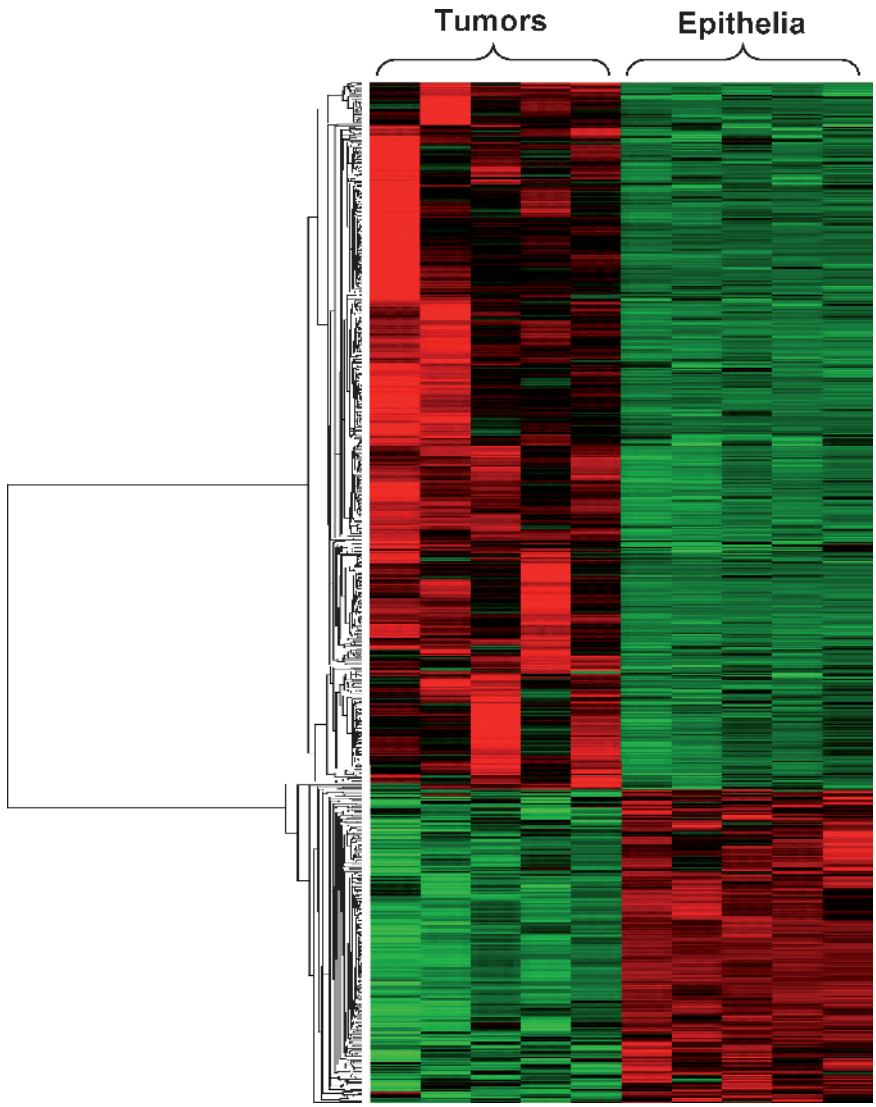

Figure 1. *Comparison of bladder epithelia and whole bladder tissues as controls to bladder cancers. Among the 1138 known genes found to be differentially expressed in rat bladder tumors compared with epithelia, 770 genes were overexpressed and 368 genes were underexpressed. Green indicates an expression below the mean value for the gene, black indicates an expression near the mean, and red indicates an expression above the mean.*

tumors. We found that inappropriate regulation of *ras*, cell cycle, and apoptosis pathways may be the three major steps in the tumorigenesis of rat bladder malignancy. In addition, we were able to identify a variety of genes whose expression was highly increased independent of whether they are directly involved in the mechanism of tumorigenesis in this model. These highly modulated genes were also proved to be changed at the protein level and may prove highly useful in identifying early lesions and tumors in samples from urine or serum. In addition, both these highly overexpressed genes and many of the genes that are along the mechanistic pathway may prove to be modulated by effective preventive or therapeutic agents.

One group of genes found to be differentially expressed in bladder tumors comprises cell cycle–related genes. Tumor proliferation depends on the derangement of normal cell cycle progression and control. Cell cycle–associated protein complexes composed of cyclins and cyclin-dependent kinases (CDKs) regulate normal cellular proliferation. Different CDK–cyclin complexes cooperate to drive cells through different phases of the cell cycle. Activation of CDK4 and CDK6 by D-type cyclins is thought to be involved in progression through early G_1. We observed an increased expression of a number of CDK-related phosphatases. Thus, a variety of cell cycle commitment genes, including *cyclins*, *CDCs*, *Cdca*, *GADD45s*, *Gas*, *Plks*, *Mad2*, and *Bub1*, were found to be overexpressed in bladder tumors. Increased levels of *cyclin D1* are associated with a wide variety of cancers, including breast, colon, and lung cancers. We confirmed the increase in *cyclin D1* observed in microarrays both by reverse transcription (RT) PCR and by IHC (Figure 3). These results help to demonstrate the potential use of array analysis in determining biomarkers that might be useful in the identification of early lesions. Overall, the results are in agreement with

Table 1. Selected Genes Whose Expression Is Upregulated in Rat Bladder Tumors Compared with Normal Bladder Epithelia Identified by Microarray.

Gene	Accession Number	Description	Fold Change	*P*
Cell cycle–related genes				
Cdc2a	NM_019296	CDC2 homolog A*	2.7*	.0250
Cdc20	U05341	CDC20 homolog*	4.6*	.0040
Cdc23	BE111697	CDC23 (cell division cycle 23, yeast, homolog) (predicted)*	33.3*	.0170
Cdc25B	NM_133572	CDC25 homolog B	2.4	.0007
Cdc42ep5	AI599324	CDC42 effector protein (rho GTPase binding) 5 (predicted)	2.6	.0027
Cdca1	BG375704	Cell division cycle–associated 1 (predicted)	5.5	.0125
Cdca2	AW532628	Cell division cycle–associated 2 (predicted)	5.6	.0456
Cdca3	BF417638	Cell division cycle–associated 3	6.1	.0298
Ccna2	AA998516	Cyclin A2*	15.3*	.0028
Ccnb1	X64589	Cyclin B1*	4.8*	.0135
Ccnb2	AW253821	Cyclin B2*	6.5*	.0041
Ccnd1	X75207	Cyclin D1*	8.8*	.0151
Cdkn2a	AF474976	Cyclin-dependent kinase inhibitor 2A ($p16^{INK4a}$)*	8.7*	.0310
Cdkn2c	NM_131902	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)*	3.7*	.0005
Cdkn2d	BI290067	Cyclin-dependent kinase inhibitor 2D*	2.2*	.0053
Cdkn3	BE113362	Cyclin-dependent kinase inhibitor 3 (predicted)*	21.3*	.0136
Mad2l1	AW143296	MAD2 (mitotic arrest deficient, homolog)–like 1 (yeast)*	2.0*	.0050
Plk1	U10188	Polo-like kinase 1 (*Drosophila*)*	3.7*	.0218
Plk4	BE109322	Polo-like kinase 4 (*Drosophila*) (predicted)*	2.5*	.0059
Gadd45b	BI287978	Growth arrest and DNA damage–inducible 45 β (predicted)*	2.7*	.0007
Gadd45g	AI599423	Growth arrest and DNA damage–inducible 45 γ (predicted)	2.4	.0106
Gas5	BF287008	Growth arrest–specific 5	2.3	.0367
Gas7	AJ131902	Growth arrest–specific 7	2.3	.0305
Gap43	NM_017195	Growth-associated protein 43	2.1	.0167
Gdf1	BI289525	Growth differentiation factor 1 (predicted)	6.1	.0769
Bub1	BF388785	Budding uninhibited by benzimidazoles 1 homolog*	11.5*	.0000
Bub1b	BF557145	Budding uninhibited by benzimidazoles 1 homolog, β	7.9	.0074
Myc	NM_012603	Myelocytomatosis viral oncogene homolog (avian)	2.5	.0077
Jundp	NM_053894 2	Jun dimerization protein 2	3.8	.0017
Dusp6	NM_053883	Dual-specificity phosphatase 6*	2.4*	.0245
Mki67	AI714002	Antigen identified by monoclonal antibody Ki-67*	20.8*	.0010
Small G proteins				
Racgap1	AI409259	Rac GTPase-activating protein 1*	17.8*	.0146
Rad51	BI303370	RAD51 homolog (*Saccharomyces cerevisiae*)*	5.1*	.0048
Rem2	BI296482	Rad- and gem-related GTP-binding protein 2	10.4	.0045
Rap2a	AW251376	Rap2A-like protein	3.6	.0190
Rap2b	NM_133410	RAP2B, member of RAS oncogene family	2.9	.0185
Rap2ip	AI535169	Rap2-interacting protein*	4.8*	.0221
Rin3	AI706777	Ras and Rab interactor 3 (predicted)	4.5	.0045
Rasgrp1	BI282819	RAS guanyl-releasing protein 1*	2.3*	.0129
Rasgrp2	AW532114	RAS guanyl-releasing protein 2 (predicted)	2.1	.0247
Arhc	AA891940	Ras homolog gene family, member C (predicted)*	2.5*	.0003
Arhd	AA955648	Ras homolog gene family, member D (predicted)*	2.1*	.0020
Arhe	AI103572	Ras homolog gene family, member E	2.8	.0158
Rnd1	AI144754	Rho family GTPase 1 (predicted)	5.1	.0405
Arhgap4	BE111827	Rho GTPase-activating protein 4	3.3	.0260
Arhgap8	AA945062	Rho GTPase-activating protein 8	2.3	.0025
	BG377320	Rho guanine nucleotide exchange factor (GEF) 17 (predicted)	2.5	.0003
Arhgdib	BF285771	Rho, GDP dissociation inhibitor (GDI) β (predicted)	3.7	.0040
Rock2	BI303031	Rho-associated coiled coil forming kinase 2	2.7	.0242
	BG378261	Rhophilin, Rho GTPase binding protein 1 (predicted)	3.9	.0128
Oncogenes and tumor-suppressor genes				
Akt1	NM_033230	V-akt murine thymoma viral oncogene homolog 1	1.5	.0077
Akt3	NM_031575	Thymoma viral proto-oncogene 3	2.4	.0255
Nf1	BM386570	Neurofibromatosis 1	3.1	.0168
Kit	AI454052	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene	4.2	.0079
Maf	NM_019318	V-maf musculoaponeurotic fibrosarcoma (avian) oncogene	2.7	.0024
Mafb	AA900536	V-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	3.1	.0004
Ect2	AI578135	Ect2 oncogene (predicted)*	12.0*	.0122
Fes	BI289400	Feline sarcoma oncogene (predicted)*	3.4*	.0068
Fyn	AI230396	Fyn proto-oncogene	2.1	.0266
Myc	NM_012603	Myelocytomatosis viral oncogene homolog (avian)	2.5	.0077
Ndr4	BG666709	N-myc downstream regulated 4	2.5	.0031
	BM390283	Large tumor suppressor 2 (predicted)	2.3	.0010
Apoptosis				
Birc2	NM_023987	Inhibitor of apoptosis protein 1*	3.3*	.0077
Birc5	NM_022274	Baculoviral IAP repeat-containing 5 (*Survivin*)	3.6*	.0500
Pawr	U05989	PRKC, apoptosis, WT1, regulator	2.0	.0003
Casp1	D85899	Caspase 1*	2.7*	.0173
Casp11	NM_053736	Caspase 11*	4.7*	.0025
Casp12	NM_130422	Caspase 12	5.7	.0010
Pycard	BI282953	Apoptosis-associated speck-like protein containing a CARD	2.1	.0047

Table 1. (*continued*)

Gene	Accession Number	Description	Fold Change	*P*
Apoptosis				
Bcl11b	BE116855	B-cell leukemia/lymphoma 11B (predicted)	4.0	.0006
Bcl2a1	NM_133416	B-cell leukemia/lymphoma 2 related protein A1*	5.6*	.0033
Bcl3	AI411774	B-cell leukemia/lymphoma 3 (predicted)*	5.8*	.0059
Bcl6	AI237606	B-cell leukemia/lymphoma 6 (predicted)*	3.6*	.0014
Bok	AI227742	Bcl-2–related ovarian killer protein*	2.2*	.0040
Cebpd	NM_013154	CCAAT/enhancer binding protein (C/EBP), δ	2.9	.0002
Tnfsf13	AA800814	Tumor necrosis factor (ligand) superfamily, member 13	2.5	.0000
Tnfip6	AF159103	Tumor necrosis factor α induced protein 6	2.4	.0135
Tnfrsf11b	NM_012870	Tumor necrosis factor receptor superfamily, member 11b*	3.9*	.0012
Tnfrsf12a	BI303379	Tumor necrosis factor receptor superfamily, member 12a	5.9	.0000
	BI278479	Tumor necrosis factor, α-induced protein 2 (predicted)	2.8	.0024
Growth factors and related genes				
Ctgf	NM_022266	Connective tissue growth factor*	7.9*	.0002
	BF284634	Epidermal growth factor–containing fibulin-like extracellular matrix protein 1	3.9	.0015
Fgf13	NM_053428	Fibroblast growth factor 13	3.6	.0007
Fgfbp1	NM_022603	Fibroblast growth factor binding protein 1*	30.2*	.0027
Hgf	NM_017017	Hepatocyte growth factor	4.3	.0022
Hgfac	BE119649	Hepatocyte growth factor activator	2.1	.0130
Hdgfrp3	BI283829	Hepatoma-derived growth factor, related protein 3	3.5	.0019
Igf1	M15481	Insulin-like growth factor 1	21.4	.0010
Igfbp3	NM_012588	Insulin-like growth factor binding protein 3*	3.2*	.0000
Igfbp4	BE108969	Insulin-like growth factor binding protein 4	2.1	.0261
Igfbp7	AI233246	Insulin-like growth factor binding protein 7	3.0	.0000
Pdgfrb	BM389426	Platelet-derived growth factor receptor, β polypeptide*	2.9*	.0017
Pdgfa	BE100812	Platelet-derived growth factor, α*	2.1*	.0044
Pdgfc	NM_031317	Platelet-derived growth factor, C polypeptide	3.0	.0054
Scgf	AI576758	Stem cell growth factor	2.2	.0055
Tgfb1	NM_021578	Transforming growth factor, β 1	6.6	.0014
Tgfb2	NM_031131	Transforming growth factor, β 2*	5.9*	.0021
Vegfc	NM_053653	Vascular endothelial growth factor C	2.5	.0039

Most of the upregulated genes were small G proteins, apoptosis genes, cell cycle–related genes, oncogenes, and growth factors. Fold change is the ratio of the mean gene expression values of tumors to the mean gene expression values of epithelia from the microarray.
*The genes were also found to be differentially expressed in mouse bladder tumors.

our finding of a relatively high proliferative index in lesions derived from this model.

Annexin A1 (ANXA1) is a calcium-binding and phospholipid-binding protein of the annexin superfamily that is found in a wide range of organisms, including vertebrates, invertebrates, and plants. Overexpression of ANXA1 was found in breast [20], stomach [21], pancreatic [22], and hepatic cancers [23], whereas underexpression of ANXA1 was recorded in prostate [24,25], esophageal [25], and head and neck [26] cancers. Thus, the role of ANXA1 in carcinogenesis may occur in a tissue-specific manner. The exact function of ANXA1 remains unknown. There are a number of possible functions of ANXA1 in cancer development. For example, ANXA1 serves as a substrate for *EGFR* [27] and is a steroid-regulated protein [28]; thus, it has been linked with cell proliferation and regulation of cell migration through the regulation of the extracellular signal–regulated kinase/mitogen-activated protein kinase (MAPK) signal transduction pathway [29]. ANXA1 is also a critical mediator of apoptosis [30]. Previous studies have also suggested that ANXA1 can serve as a gene target and gene maker for cancer treatment, development, progression, and diagnosis. Expression of ANXA1 significantly correlated with clinicopathological features and survival in esophagus and esophagogastric junction adenocarcinomas [31] and breast cancer [32]. Immunocytochemical detection of ANXA1 represents a simple, inexpensive, highly sensitive, and specific assay for the diagnosis of hairy cell leukemia [33].

The striking increases in *ANXA1* levels observed in the microarray, which was confirmed by RT-PCR, were then examined by 2D gel analysis (Figure 3). Finally, based on consistent increases observed in these various studies, we performed IHC and similarly found striking increases in annexin A1 expression. As can be seen in the IHC panel, a strong response should allow us to examine the expression of annexin A1 for identifying earlier lesions (dysplasias and papillomas) or to potentially look for it in the urine of tumor-bearing rats. Thus, the results with cyclin D1 and annexin A1 demonstrate methods that might allow one to combine results from gene expression and proteomic analyses.

We also found that *COX-2* expression was increased by roughly five times in RNA expressions in bladder tumors. Interestingly, by IHC, we have shown that the highest expression was not in epithelial cells but rather in endothelial cells in the tumor [3]. Furthermore, we have found that both celecoxib and a wide variety of nonsteroidal anti-inflammatory drugs (NSAIDs) were highly effective in blocking bladder tumor formation in this model [3]. This parallels epidemiologic studies in humans showing the efficacy of NSAIDs against bladder tumors. Interestingly, levels of *PPARγ* were reduced roughly by 3.5 times in microarrays. We have recently found that PPARγ expression was

decreased in tumors, as assessed by IHC procedures (Grubbs and Fischer, data not shown). That indirectly reflects our finding that the PPARγ agonist rosiglitazone is a bladder tumor promoter in this model. Two mechanistically significant genes that we have previously demonstrated to exhibit altered levels in rat bladder tumors are *Fhit* (fragile histidine triad) and the IAP (inhibition of apoptosis protein) *Survivin*. *Fhit* expression is often lost in a wide variety of human tumors (e.g., lung, head and neck, and bladder). We have previously shown that the *Fhit* gene is methylated in rat bladder tumors and is associated with a decrease in FHIT protein expression [34]. We also previously found an increased expression of the IAP protein in rodent bladder tumors [35], as it is expressed in a variety of human tumors, including bladder. These results reinforce the use of microarrays in looking for the expression of specific "relevant genes," in addition to its more generalized use in looking for important pathways (Figure 4, *A*–*D*) or a much wider variety of genes (Tables 1 and 2).

Table 2. Selected Genes Whose Expression Is Downregulated in Rat Bladder Tumors Compared with Normal Bladder Epithelia Identified by Microarray.

Gene	Accession Number	Description	Fold Change	*P*
Cell cycle–related genes				
	BE120410	Cyclin M1 (predicted)	−2.7	.0110
Cdkn1c	AI013919	Cyclin-dependent kinase inhibitor 1C, p57	−5.8	.0000
Jundp1	NM_021865	Jun dimerization protein 1	−2.1	.0024
Ppara	NM_013196	Peroxisome proliferator-activated receptor α*	−2.2*	.0153
Pparg	NM_013124	Peroxisome proliferator-activated receptor, γ*	−2.3*	.0003
Small G proteins				
Rab14	AA875010	RAB14, member RAS oncogene family	−2.1	.0126
Rab27b	NM_053459	RAB27B, member RAS oncogene family	−3.3	.0000
Rab3c	NM_133536	RAB3C, member RAS oncogene family	−6.7	.0002
Rab40b	AA924620	RAB40b, member RAS oncogene family (predicted)	−3.2	.0001
	BF284067	RAP1, GTPase-activating protein 1 (predicted)	−3.6	.0000
Rasa3	AI237779	RAS p21 protein activator 3	−2.6	.0014
RICS	BE097238	RhoGAP involved in β-catenin–N-cadherin and NMDA receptor signaling (predicted)	−2.1	.0005
	AI547942	RhoGEF (Arhgef) and pleckstrin domain protein 1	−2.2	.0009
Oncogenes and tumor-suppressor genes				
	AA900477	Vav2 oncogene (predicted)	−2.3	.0002
Mycl1	BI300996	V-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma–derived (avian)*	−2.2*	.0001
Ndrg2	NM_133583	N-myc downstream-regulated gene 2*	−3.0*	.0000
	BE115673	Metastasis suppressor 1 (predicted)	−4.7	.0013
Pak3	NM_019210	P21 (CDKN1A)–activated kinase 3	−14.3	.0000
Pak4	BF404920	P21 (CDKN1A)–activated kinase 4 (predicted)	−3.3	.0130
Wt1	NM_031534	Wilms tumor 1	−2.7	.0278
Brca2	BF396613	Breast cancer 2	−3.5	.0161
	AI548958	HRAS-like suppressor (predicted)	−2.5	.0015
Fhit	NM_021774	Fragile histidine triad gene	−1.6*	.0107
Apoptosis				
Dffa	NM_053679	DNA fragmentation factor, α subunit*	−2.0*	.0060
LOC64171	NM_022303	Caspase recruitment domain protein 9	−2.0	.0012
Ntrk1	NM_021589	Neurotrophic tyrosine kinase, receptor, type 1	−3.5	.0391
Growth factors				
	BF418373	Epidermal growth factor–like protein 6	−8.3	.0000
Fgf1	BI289840	Fibroblast growth factor 1*	−2.8*	.0006
FGFR2	L19107	Fibroblast growth factor receptor 2	−2.9	.0384
	BE112403	Fibroblast growth factor receptor substrate 2 (predicted)	−2.0	.0042
	BF396448	Insulin-like growth factor binding protein–like 1	−2.2	.0036
	BM384311	Platelet-derived growth factor receptor–like (predicted)	−4.4	.0000
Others				
Csnk1g1	AA957549	Casein kinase 1, γ 1	−2.1	.0058
Csnk1g3	AI176776	Casein kinase 1, γ 3	−2.5	.0147
	BE107780	Casein kinase II, α 2, polypeptide (predicted)	−2.3	.0012
Clpx	BG371721	Caseinolytic protease X (*Escherichia coli*) (predicted)	−2.7	.0000
Cyp3a18	D38381	Cytochrome *P*450, 3a18	−5.6	.0001
Cyp1a1	X00469	Cytochrome *P*450, family 1, subfamily a, polypeptide 1	−22.1	.0053
Cyp11a1	NM_017286	Cytochrome *P*450, family 11, subfamily a, polypeptide 1	−3.6	.0000
Cyp4a14	AA893326	Cytochrome *P*450, family 4, subfamily a, polypeptide 14	−5.2	.0487
Lcmt1	BG381002	Leucine carboxyl methyltransferase 1	−2.1	.0001
	BE107055	Leucine-rich repeat-containing 28 (predicted)	−2.0	.0001
	AI716087	Leucine zipper transcription factor–like 1 (predicted)	−2.0	.0000
	BF412229	Leucine-rich and death domain–containing (predicted)	−2.2	.0001
Lgi1	AI229354	Leucine-rich, glioma-inactivated 1	−3.7	.0007
	AI235414	RAR-related orphan receptor α (predicted)	−2.4	.0000
	BE110171	RAR-related orphan receptor γ (predicted)	−2.0	.0125

Downregulated genes include Rab subfamily genes, tumor-suppressor genes, casein kinases, cytochrome *P*450s, and RAR-related orphan receptor genes. Fold change is the ratio of the mean gene expression values of tumors to the mean gene expression values of epithelia from the microarray.
*The genes were also found to be differentially expressed in mouse bladder tumors.

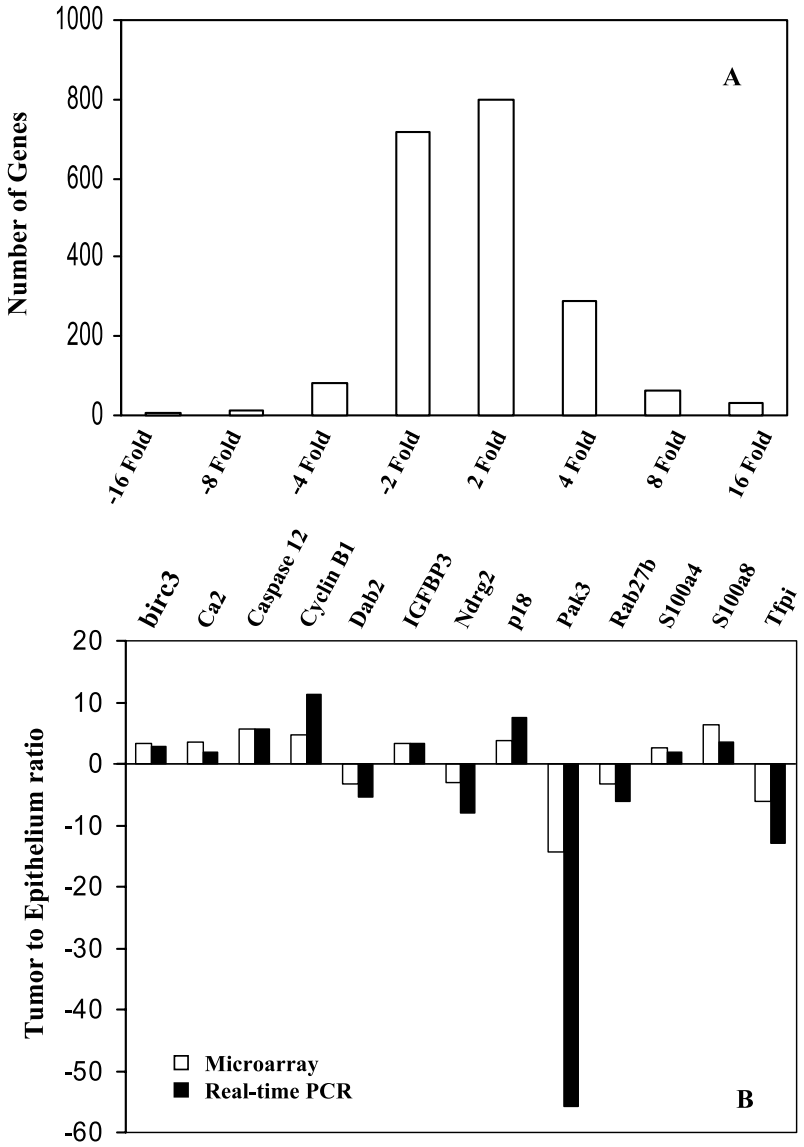

Figure 2. *Distribution of 2005 differentially expressed genes and ESTs by microarray analysis and real-time PCR confirmation for selected genes. (A) An overview of the number of genes reveals fold changes different from those in normal bladder epithelia. (B) Comparison of fold change produced by microarray with the relative expression ratio obtained from real-time PCR, with good concordance.*

The *ras* superfamily regulates many cellular processes, such as cell cycle progression, actin cytoskeletal dynamics, and membrane traffic. The transforming potential of *ras* is due to a mutation, which, in human bladder tumors, occurs in H-*ras* [36], although these rat tumors do not appear to have mutations in *ras* genes. Alternatively, overexpression of H-*ras*, K-*ras*, and N-*ras* transcripts has also been associated with bladder tumor transition [37,38]. Guanine nucleotide exchange factors (GEFs) stimulate Ras superfamily members to exchange bound guanosine 5c-diphosphate (GDP) for guanosine 5c-triphosphate (GTP), thereby increasing the amount of active form [39]. *Rho* mutations in tumors are quite rare, but overexpression is more common [40]. Dysregulation of Rho family member activity probably also contributes to human cancer in that some *RhoGEFs* act as oncogenes [41], whereas *RhoGAPs* [42] act as tumor suppressors. Reduced expression of *RhoGDIs* has recently been shown to correlate with increasing invasive and metastatic abilities in human bladder carcinoma cell lines [43,44]. We found that *RhoGAPs*, *RhoGEFs*, and *RhoGDIs* were differentially expressed in rat bladder tumors.

Our results implicated many members of the integrin-mediated cell adhesion pathway in bladder tumorigenesis. *Integrin* $\alpha 7$, *calpain 9*, *Pak3*, *rhodopsin* (*Rho*), *Rap1a*, and *Vav2* underexpressed rat bladder tumors, whereas *integrin* β_4, *actin* $\alpha 1$, *caveolin-2*, *Fyn*, *Rock2*, *Mylk2*, and *Akt3* overexpressed in rat bladder tumors. The small GTPase Rap1 is involved in several aspects of cell adhesion, including integrin-mediated cell adhesion and cadherin-mediated cell junction formation [45]. Rap1 regulates all integrins that are associated with the actin cytoskeleton, such as integrins β_1, β_2, and β_3 [46]. Active Rap1 binds to a subset of Rac

GEFs, including Vav2 and Tiam1. Overexpressed Vav2 and Tiam1 specifically require Rap1 to promote spreading, even though Rac1 is activated independently of Rap1 [47]. Rac is both required and sufficient to mediate Rap1-induced cell spreading. Thus, integrin-mediated cell adhesion appears to play a role in rat bladder tumorigenesis and progression.

Increased activity of another Ras effector, PI3-kinase, is similarly associated with many types of human cancer. Because PI3-kinase is an immediate downstream effector of Ras and EGFR, multiple pathways may contribute to the increases in PI3-kinase activity observed in many bladder cancers. PI3-kinase consistently prevents apoptosis in many cell systems through activation of the Rac GTPase, possibly through activation of NF-κB [48]. Thus, the activation of PI3-kinase associated with excessive Ras activity may promote oncogenesis by blunting apoptosis-inducing stimuli associated with oncogenic transformation. RacGAPs stimulate intrinsic GTPase activity, thus leading to Rac inactivation. Rac and Rac-GEFs play key roles in the control of various aspects of malignant transformation and metastatic cascade in various models [49], as well as in the control of mitogenesis through its ability to regulate G_1/S transition and cyclin D1 expression [50–52]. Ect2 and RacGAP also regulate the activation and function of Cdc42 in mitosis [53].

Finally, the differentially expressed genes between mouse and rat bladder tumors were compared. We found that, among the 860 comparable genes between rats and mice, there were 280 genes that had the same tendency of expression in both mouse and rat bladder tumors, accounting for about one third of the genes. The majority of these genes are cell cycle–related genes, ras small G proteins, and apoptosis-related and growth factor–related genes. These results strongly

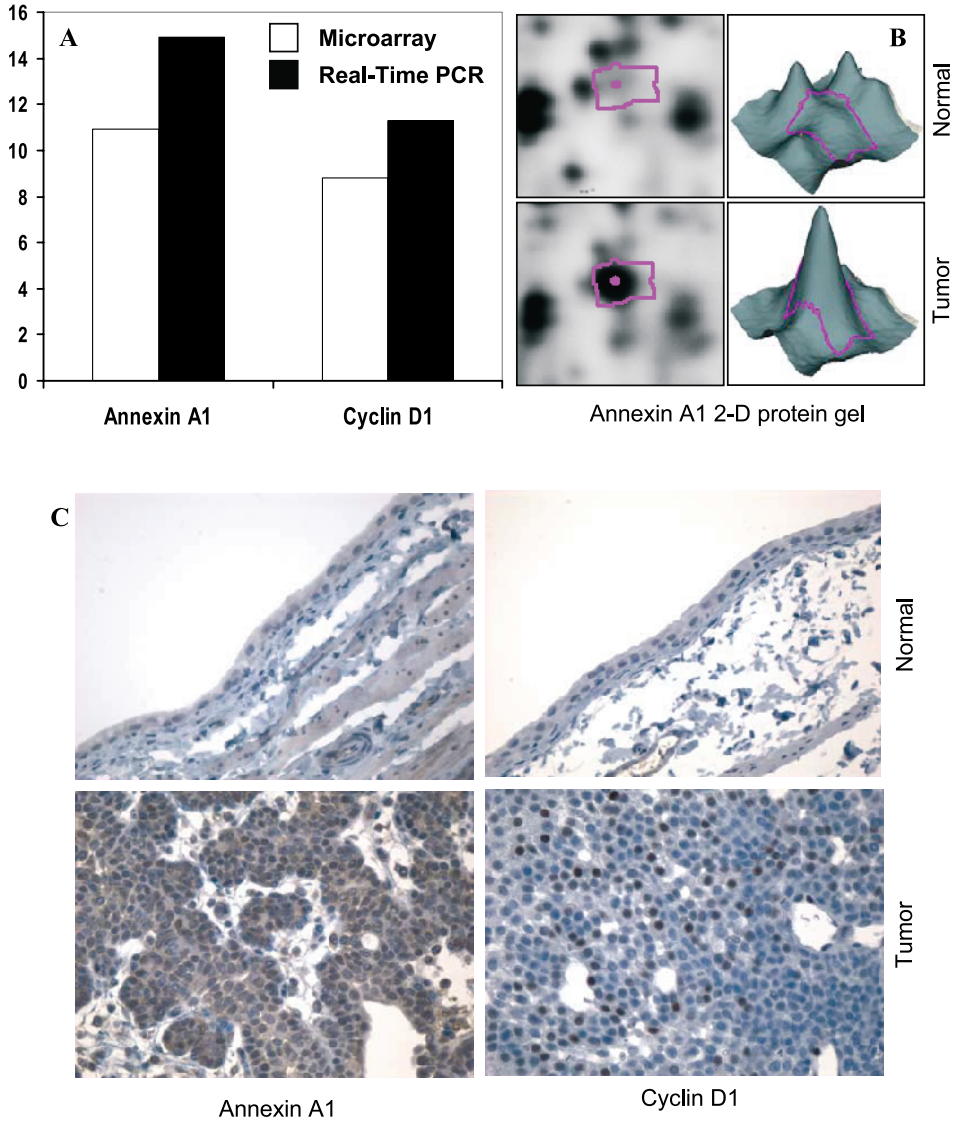

Figure 3. *Confirmation of differentially expressed genes for both RNA and protein levels. (A) Comparison of fold change produced by microarray with the relative expression ratio obtained from RT-PCR. (B) 2D protein gel electrophoresis indicates that annexin A1 protein is overexpressed in rat bladder cancers. (C) IHC suggests that both annexin A1 and cyclin D1 are overexpressed in rat bladder cancers.*

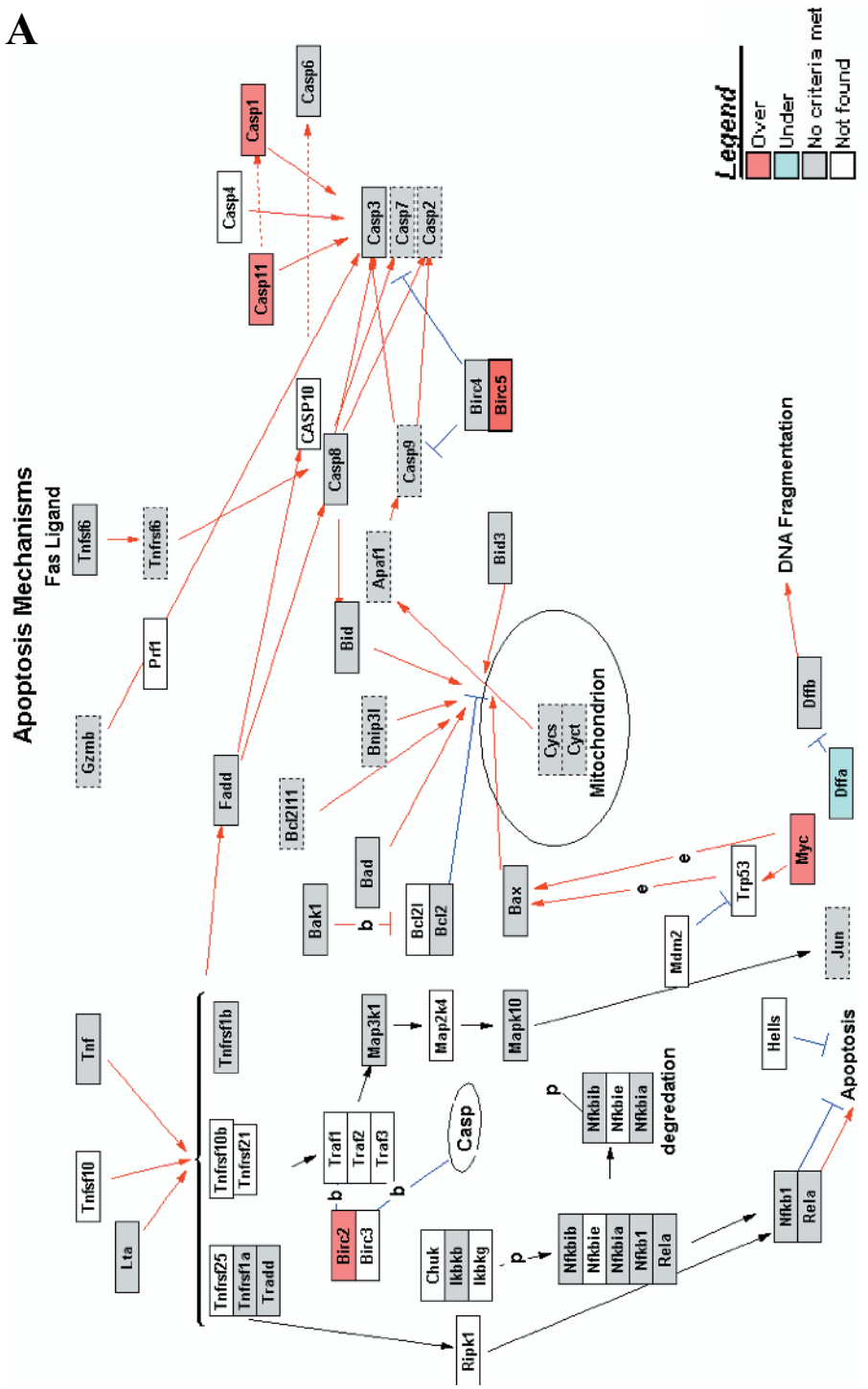

Figure 4. *GenMAPP signaling pathways integrated into rat bladder tumorigenesis with a cutoff fold change of ≥ 1.5 and P < .05. Yellow and blue indicates overexpressed and underexpressed genes in tumor samples, respectively. Grey indicates that selection criteria were not met but the gene was represented in the array. White boxes indicate that the gene was not present in the chip. (A) Apoptosis. (B) Cell cycle. (C) G_1-to-S cell cycle control. (D) Integrin-mediated cell adhesion.*

indicated that both in mice and in rats, similar mechanisms are involved in bladder tumorigenesis.

In human bladder tumors, low-grade papillary tumors frequently show constitutive activation of the receptor tyrosine kinase/Ras pathway, exhibiting activating mutations in H-*ras* and fibroblast growth factor receptor 3 (*FGFR3*) genes [54,55]. In contrast, carcinoma in situ and invasive tumors frequently show alterations in *p53* and *Rb* genes and pathways [56,57]. The cell cycle is controlled by the p53 and Rb pathways, with cells receiving extracellular growth signals through the Ras/MAPK pathway. Previous studies have shown that chemically induced rat bladder cancers typically have a mixed histology showing elements of both transitional and squamous cells [3,4], with a relatively low

frequency of H-*ras* mutations and roughly 50% of these tumors developing *p53* mutations [58,59], similar to the percentage found in humans.

In recent years, genomewide expression profiling by the use of microarray technology has provided new insights into the gene expression patterns and dysregulation of genes during bladder tumorigenesis. Gene expression profiles can be used not only to elucidate the underlying molecular mechanisms and pathways involved in bladder tumorigenesis but also to make distinctions among different histologic subtypes and to predict tumor recurrence and patient survival. Gene expression array studies on human bladder cancers have revealed a broad range of genes that are differentially expressed during bladder tumorigenesis, progression, and invasion. Kawakami et al. [60] indicated that genes involved in metabolism, transcription, cell adhesion/

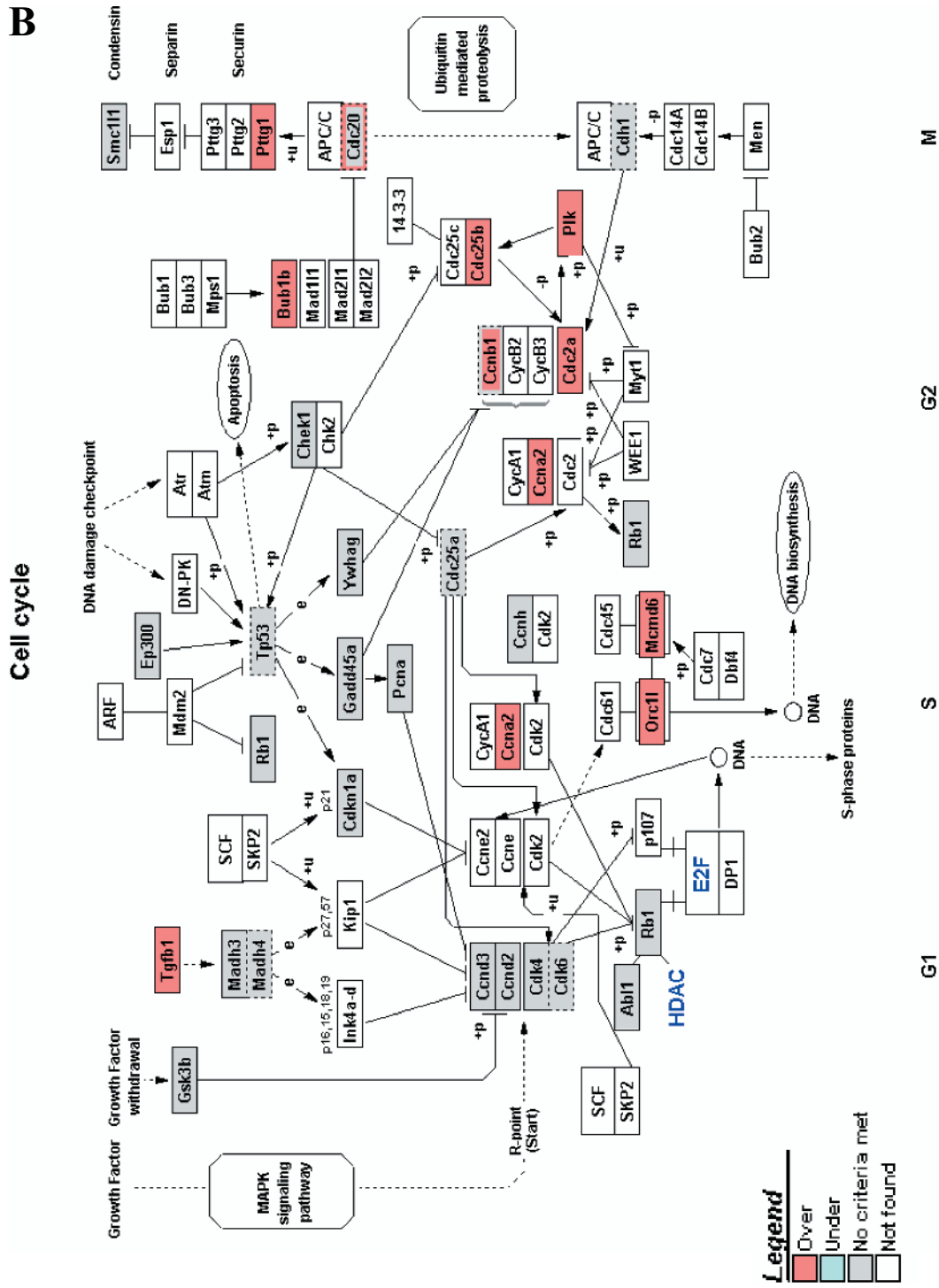

Figure 4. *(continued)*

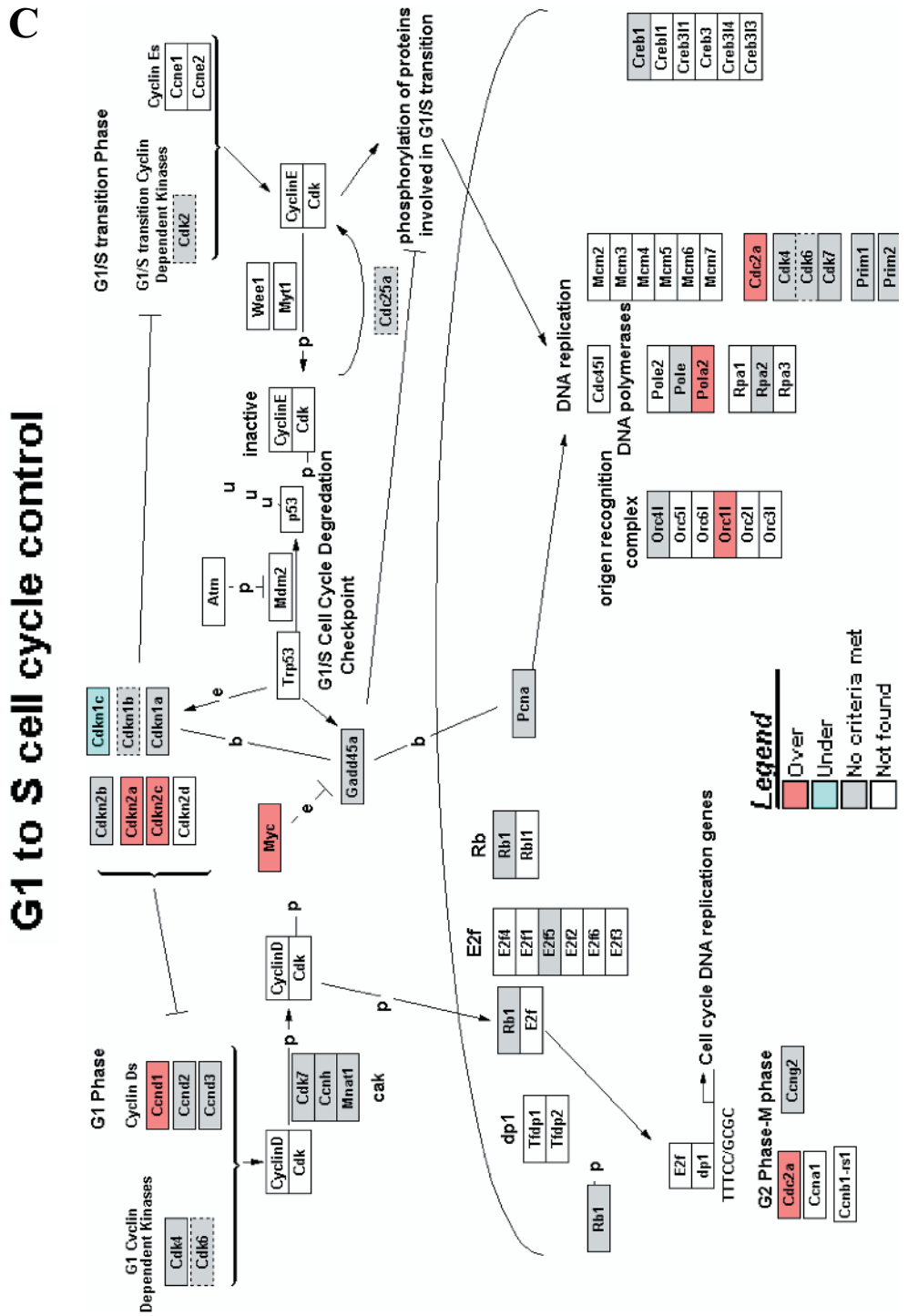

Figure 4. *(continued)*

surface, and cytoskeleton/cell membrane were significantly differentially expressed in superficial and invasive bladder tumors. Kim et al. examined gene expression patterns in the development of bladder cancer from preneoplasia along papillary and nonpapillary pathways and identified alterations in seven gene clusters controlling proliferation, differentiation, and apoptosis that were common for both papillary and nonpapillary cancers. In contrast, genes controlling cellular and stromal interactions were altered in nonpapillary cancer [61]. Elsamman et al. also identified the significant upregulation of 40 genes in cell differentiation and keratinization, cell cycle, cell adhesion, transcription, and apoptosis associated with superficial noninvasive bladder tumors, and the significant upregulation of 34 genes related to extracellular matrix degradation, immune responses, cell cycling, and angiogenesis was associated with invasive bladder tumors [62]. Dyrskjot et al. identified a 45-gene signature of bladder tumor progression that was involved in regulating apoptosis,

cell differentiation, and cell cycle. *BNIP3L*, *BIRC4*, *NCKAP1*, and *BIRC6* genes, which are involved in the apoptotic cell death pathway, were upregulated in the nonprogressing group. *Cdc25B*, *Cdc20*, and *MCM7* genes, which are involved in regulating cell cycle and cell proliferation, were upregulated in the progressing group [63]. Modlich et al. also identified genes encoding transcription factors, protein synthesis, and metabolism; cell cycle progression and differentiation were overexpressed in superficial bladder tumors, whereas transcripts for immune, extracellular matrix, adhesion, peritumoral stroma, and muscle tissue components; proliferation; and cell cycle controllers were upregulated in invasive tumors [64]. In concordance with human bladder tumors, in this study, we confirmed chemically induced rat bladder cancers undergoing similar molecular mechanisms and pathways during tumorigenesis and,

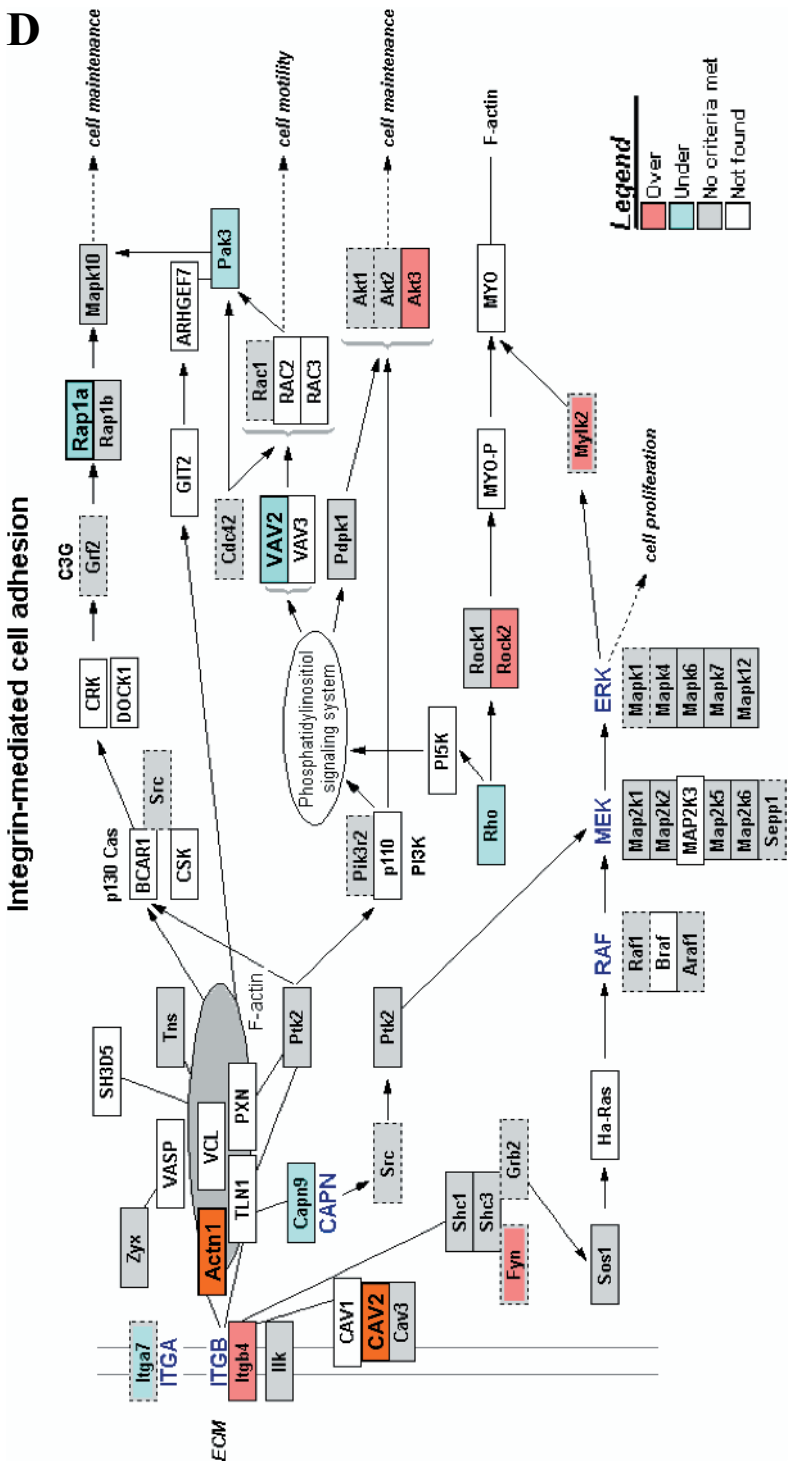

Figure 4. *(continued)*

maybe, in progression. Genes involved in cell cycle, apoptosis, cell adhesion, transcription factors, and *ras* gene pathway were significantly differentially expressed in rat bladder cancers (Tables 1 and 2). These results suggested that chemically induced rat bladder cancers can be used to represent human bladder cancer. It is a good model for studying the pathogenesis, progression, treatment, and prevention of human bladder cancer.

In summary, we have determined the expression profiles of genes differentially expressed during rat bladder tumorigenesis. Our results suggest that EGFR-Ras pathway, cell cycle, apoptosis, and integrin-mediated cell adhesion are involved in bladder tumorigenesis. Our results also suggest that common mechanisms play important roles in both rat and mouse tumorigeneses. Furthermore, certainty on identified genes may suggest potential target molecules for preventing cancer in this model.

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