[CANCER RESEARCH 63, 179–185, January 1, 2003]

Allelic Loss of p53 Gene Is Associated with Genesis and Maintenance, but not Invasion, of Mouse Carcinoma *in Situ* of the Bladder[1]

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ABSTRACT

Carcinoma *in situ* (CIS) of the bladder has recently been proposed to be a heterogeneous group of diseases with varied histogenesis and biological behavior. In this study, we describe the sequential steps of CIS development and progression in a transgenic mouse model expressing low levels of the SV40 large T antigen. We found that CIS in transgenic mice arose from urothelial dysplasia, that CIS could persist for an extended period of time without invasion, and that the majority of CIS eventually evolved into high-grade, superficial, papillary tumors before a small fraction of them advanced to invasion/metastasis. A genome-wide search of chromosomal imbalances by comparative genomic hybridization revealed that 9 of 11 (82%) of CIS had losses on chromosome 11. Southern blotting demonstrated the allelic loss of the p53 gene, which resides on mouse chromosome 11, in four comparative genomic hybridization-tested tumors and 10 of 11 (91%) additional CIS examined. Consistent with the reduced p53 gene dosage because of the allelic loss and the functional inactivation of p53 protein of the remaining allele by SV40T antigen, there was a dramatic decrease in CIS of Mdm-2, a major p53 target. In contrast, the level of p21, another p53 target, was largely unaltered, suggesting that p21 expression can be regulated by p53-independent mechanisms. These results delineate the early stages of bladder tumorigenesis and suggest that the loss of a p53-bearing chromosome is an early event in bladder tumorigenesis and is crucial for the genesis and the maintenance, but not the progression, of bladder CIS. On the basis of our current and previous transgenic studies, we have proposed an integrated pathway progression model of bladder cancer.

INTRODUCTION

Human CIS[3] of the bladder is defined as a flat intraurothelial lesion comprised of cytologically malignant cells (1–5). Although innocuous in appearance, CIS is generally considered to have an aggressive biological behavior by frequently invading into bladder lamina propria and muscularis (6). It has been suggested that CIS is a precursor of invasive bladder cancer and requires close monitoring and intervention upon diagnosis. However, important questions remain regarding the natural course of CIS genesis and progression. Most (90%) of the CIS lesions are in fact identified in association with high-grade, invasive tumors. Primary CIS, those occurring without concurrent or preceding invasive carcinomas, are much less common (7). It is unclear whether these two morphologically similar forms of CIS represent the sequential steps in progression or the two biologically different entities (8). It is also unclear whether CIS can directly advance to invasive tumors or if there is an intermediary. Furthermore, some primary CIS lesions, although perhaps in small percentage, can remain indolent over an extended period of time. Suggestions have therefore been made that CIS may be a heterogeneous group of diseases with variable potentials of invasion (7, 8).

Only a few studies have been devoted to address the genomic alterations associated with human CIS. Spruck *et al.* (9) found 65% (15 of 23 cases) of CIS containing p53 gene mutation and 27% with allelic loss of human chromosome 17, which harbors the p53 gene. In striking contrast, these alterations were detected in only 3% of the low-grade, superficial papillary tumors. The latter tumors, however, harbored frequent (34%) chromosome 9 deletions, which were infrequent (12%) in CIS (9). Rosin *et al.* (10) examined the partial allelotype of 31 cases of microdissected CIS and detected the LOH of chromosome 17p in 60% of the cases examined, thus confirming the relationship between allelic loss of chromosome 17 and CIS formation. However, these investigators also found LOH of chromosomes 9 and 14 in as many as 77 and 70% of the CIS, respectively, along with other LOH involving 8p (65%), 13q (56%), 11p (54%), and 4p (52%). These results led to the suggestion that CIS may already have acquired the genetic alterations possessed by the invasive tumors. Using fluorescence *in situ* hybridization, Hartmann *et al.* (11) recently examined 33 cases of CIS and found almost identical deletion frequencies on chromosomes 9 (86%) and 17 (84%). Data from these latter two studies seem to indicate that chromosome 9 alterations cannot differentiate low-grade superficial papillary tumors from CIS, whereas chromosome 17 deletions are more specific for CIS.

We have recently developed transgenic mouse models to better understand the histogenesis and genetic alterations of CIS. By specifically expressing oncogenes in the urothelium using the uroplakin gene promoter, we have generated two transgenic mouse models of bladder tumorigenesis. The first expressed an activated Ha-ras oncogene that induced urothelial hyperplasia, some of which over time progressed to form low-grade, superficial papillary tumors (12). The second model that expressed the SV40 large T antigen exhibited several courses of bladder tumorigenesis depending on the copy number of the transgene (13). Those harboring 6–10 copies developed invasive and metastatic bladder cancers and succumbed to death at 3–5 months of age, whereas those harboring only 1–2 copies consistently developed bladder CIS at early ages. This raised the interesting possibility that the low-copied SV40T transgenic mice would follow a more stochastic and more physiologically relevant sequence of tumor progression. In addition, because the SV40T transgenic mice were generated in an inbred (FVB/N) strain thus having homogeneous genetic background and because CIS phenotypes occurred with 100% penetrance, these mice provide unique opportunities for prospective studies and genetic analysis of CIS (13).

In this study, we performed a systematic histopathological analysis of the sequential steps before and after CIS formation in the SV40T transgenic mice. We also carried out a genome-wide scanning of chromosomal imbalances with a focus on CIS, using CGH. In addition, we studied the allelic loss of the p53 gene and the functional

Received 8/5/02; accepted 10/24/02.

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[1] This study was supported, in part, by a merit review grant from Veterans Administration Research Service and NIH R01 Grant DK56903 (to X-R. W.) and NIH Grant DK52206 to (T-T. S.).

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[3] The abbreviations used are: CIS, carcinoma *in situ*; LOH, loss of heterozygosity; CGH, comparative genomic hybridization; DOP-PCR, degenerate oligonucleotide primer-PCR; MAPK, mitogen-activated protein kinase; EGFR, epidermal growth factor receptor.

status of p53 target genes in CIS. Our results provide important new information about CIS genesis and progression and shed new light on the genetic alterations critical for CIS maintenance. These studies, combined with our previous transgenic work, enabled us to propose an integrated model of bladder cancer formation and progression.

MATERIALS AND METHODS

Histopathology of UPII/SV40T Transgenic Mice. Of the four transgenic lines that harbored the UPII/SV40T transgene (13), line F19 harbored two copies of the transgene, did not succumb to death at early ages, and was therefore used for tumor progression analyses. Mouse breeding was carried out within the FVB/N (inbred) strain throughout to ensure homogeneous genetic background. Transgenic mice that were heterozygous for the transgene were sacrificed sequentially from birth to 16 months of age. Their urinary bladders were dissected and fixed in 10% formalin for paraffin embedding. Three-μm sections were routinely processed for H&E staining and were examined microscopically for pathological changes.

Extraction of Genomic DNA. Genomic DNA was isolated from the urothelia of individual transgenic animals using a protease K digestion and NaCl precipitation method. Briefly, freshly dissected urinary bladders were inverted, and their mucosal lining was scraped off with a chemical spatula. Microscopic examination showed that the scraped bladders were largely devoid of urothelial layers and that the scraped cell suspension was highly enriched in tumor cells with little stromal cell contamination. After washing in PBS, the tumor cells were digested at 55°C overnight with protease K solution containing 50 mM Tris/HCl (pH 7.4), 100 mM EDTA, 100 mM NaCl, 0.5% SDS, and 0.4 mg/ml protease K. Proteins were precipitated by the addition of 6 M NaCl solution to a final concentration of 1.2 M. After centrifugation, genomic DNA in the supernatant was precipitated by ethanol. The precipitates were dissolved in Tris-EDTA buffer (pH 8.0) containing 50 μg/ml RNase. For visible bladder tumors, the papillary lesions were excised for DNA extraction. Normal control DNA was extracted from tail biopsies of the wild-type mice. The majority of tumor samples (12 of 14) from single mouse bladders yielded sufficient amounts of genomic DNA for direct CGH analysis, except for two samples for which PCR amplification was performed using DOP-PCRs (14).

CGH. Tumor DNA and normal DNA were labeled with fluorescein-12-dUTP (NEN, Boston, MA) and Alexa 568-5-dUTP (Molecular Probes, Eugene, OR), respectively, by nick-translation (14–16). Inverse hybridization was performed with reverse labeling for all samples. Equal amounts (800 ng) of labeled tumor and normal DNA were mixed, coprecipitated with 40 μg of mouse Cot-1 DNA (Invitrogen, Carlsbad, CA), and resuspended in 10 μl of hybridization mix (50% formamide, 10% dextran sulfate, 2× SSC). The mixture was heated for 15 min at 70°C to denature the DNA, hybridized to normal metaphase spreads from C57 Black lymphocytes embryonic fibroblasts, and incubated at 37°C for 2 days. Slides were washed three times in 50% formamide-2× SSC at 45°C and once each in 2× SSC at 45°C, 2× SSC at room temperature, 4× SSC/0.1% Triton X-100 at room temperature, and H_2O. Slides were counterstained with 0.1 μM 4′,6′-diamino-2-phenylindole. After digital image capture of chromosome spreads, the CGH profiles comparing fluorescence intensities were analyzed using QUIPS software (Vysis/Applied Imaging, Abbott Park, IL). Thresholds for discerning gains and losses were set at 1.25 and 0.85, respectively.

Southern Blot Analysis. Genomic DNA isolated from urothelial carcinomas of the UPII/SV40T transgenic mice were digested with *Bam*HI, gel-electrophoresed, and transferred onto a nylon membrane, which was probed with a ^{32}P-labeled *Kpn*I fragment of mouse p53 cDNA from plasmid LR10 (courtesy of Dr. Larry Donehower of Baylor College of Medicine; Ref. 17). The probe was known to react with exons 2–6 of the mouse p53 gene. DNA from wild-type mice and p53 knockout mice (both heterozygotes and homozygotes; The Jackson Laboratory, Bar Harbor, ME) were similarly digested and used as controls for semiquantitative purposes.

Western Blot Analysis. Normal urothelial and tumor cells were dissolved in a lysis buffer containing 10% SDS, 20 mM Tris/HCl (pH 7.4), 50 mM NaCl, 5 mM β-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The mixture was centrifuged at 12,000 × *g* for 15 min, and the soluble proteins were quantified using the Bradford method (Bio-Rad, Hercules, CA; Ref. 18). One hundred μg of the total proteins were resolved in 8% SDS-PAGE and electrotransferred onto Immobilon-polyvinylidene difluoride membrane (Millipore, Bedford, MA). After incubation with primary and secondary antibodies, the membrane was developed with an enhanced chemiluminescence method (NEN, Boston, MA). The primary antibodies were: anti-p53 (1:2000; Oncogene Research Products, Cambridge, MA), anti-pRb (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Mdm-2 (1:200; Santa Cruz Biotechnology), anti-p21 (1:200; Santa Cruz Biotechnology), antiphosphorylated MAPK (1:1000; New England Biolabs, Inc., Beverly, MA), and anti-MAPK (1:500, New England Biolabs).

Immunohistochemistry. Deparaffinized tissue sections were microwaved in citrate buffer (pH 6.0) for 15 min for antigen retrieval and were then stained with anti-p53 and anti-p21 antibodies (see above) followed by secondary antibodies conjugated with peroxidase. The sections were counterstained with Myers' hematoxylin.

RESULTS

Genesis and Progression of Bladder CIS in UPII/SV40T Transgenic Mice. We previously generated four lines of UPII/SV40T transgenic mice, which exhibited noticeably different courses of tumor development depending on the transgene dosage (13). The two lines that harbored 6–10 copies of the transgene developed invasive and metastatic urothelial carcinomas at 3–5 months of age and rapidly succumbed to death. In contrast, the other two lines each harboring 2 copies of the transgene were highly viable, thus providing a unique opportunity for dissecting the sequential steps of tumor progression. Mice from one of the latter two lines (F19) were therefore sacrificed at different ages, and the histopathology of the urinary bladders was determined. As shown in Fig. 1*A*, the bladders of newborn, nontransgenic mice exhibited large mucosal folds protruding into the lumen, a common feature in contracted bladders of newborn and adult mice. Urothelia of the newborn animals were very thin, consisting of only two to three layers of small-sized and well-polarized cells (Fig. 1*A*, *inset*). In contrast, urothelia of newborn transgenic mice were dysplastic, with nuclear pleomorphism, hyperchromatism, and increased nuclear/cytoplasmic ratio (Fig. 1*B*, *inset*). These nuclear abnormalities were, however, primarily confined to the superficial umbrella cells, presumably because of more active transcription of the uroplakin II promoter and subsequently more SV40T expression in these cells. Nevertheless, cell layer increase, loss of epithelial polarity, and nuclear crowding were minimal. All but two newborn mice (19 of 21) had urothelial dysplasia (Fig. 1*B*; Table 1). Between 1 and 5 months, urothelia became much thicker, exhibiting profound nuclear pleomorphism, crowding, and complete loss of cell polarity (Fig. 1, *D–F*). Giant nuclei and mitotic figures were frequently seen. Nevertheless, the basement membranes remained intact. These features strikingly resembled CIS in humans. The CIS in transgenic mice invariably affected the entire bladder urothelium, which was consistent with the fact that SV40T antigen was expressed in all urothelial cells (13). The majority of the mice (21 of 24) sacrificed between 6–10 months of age remained at the CIS stage, suggesting that these lesions had a relatively long latency before progression. Between 10–16 months, 8 of 12 (67%) of the mice developed large superficial papillary tumors, the majority (6 of 12) of which were of moderate to high pathological grade (Fig. 1, *G* and *H*). Two mice had poorly differentiated urothelial carcinomas that metastasized to lymph nodes, liver, and lungs (Fig. 1, *I* and *J*). Taken together, these data indicate that tumor progression in UPII/SV40T transgenic mice followed sequential steps of dysplasia, CIS, high-grade superficial papillary tumors, and invasive/metastatic tumors and that CIS can persist for an extended period before progressing into invasive tumors.

Identification of Genomic Alterations by CGH. CGH was performed on 11 cases of CIS and 3 cases of high-grade superficial papillary tumors. Because dysplasia occurred exclusively in newborn

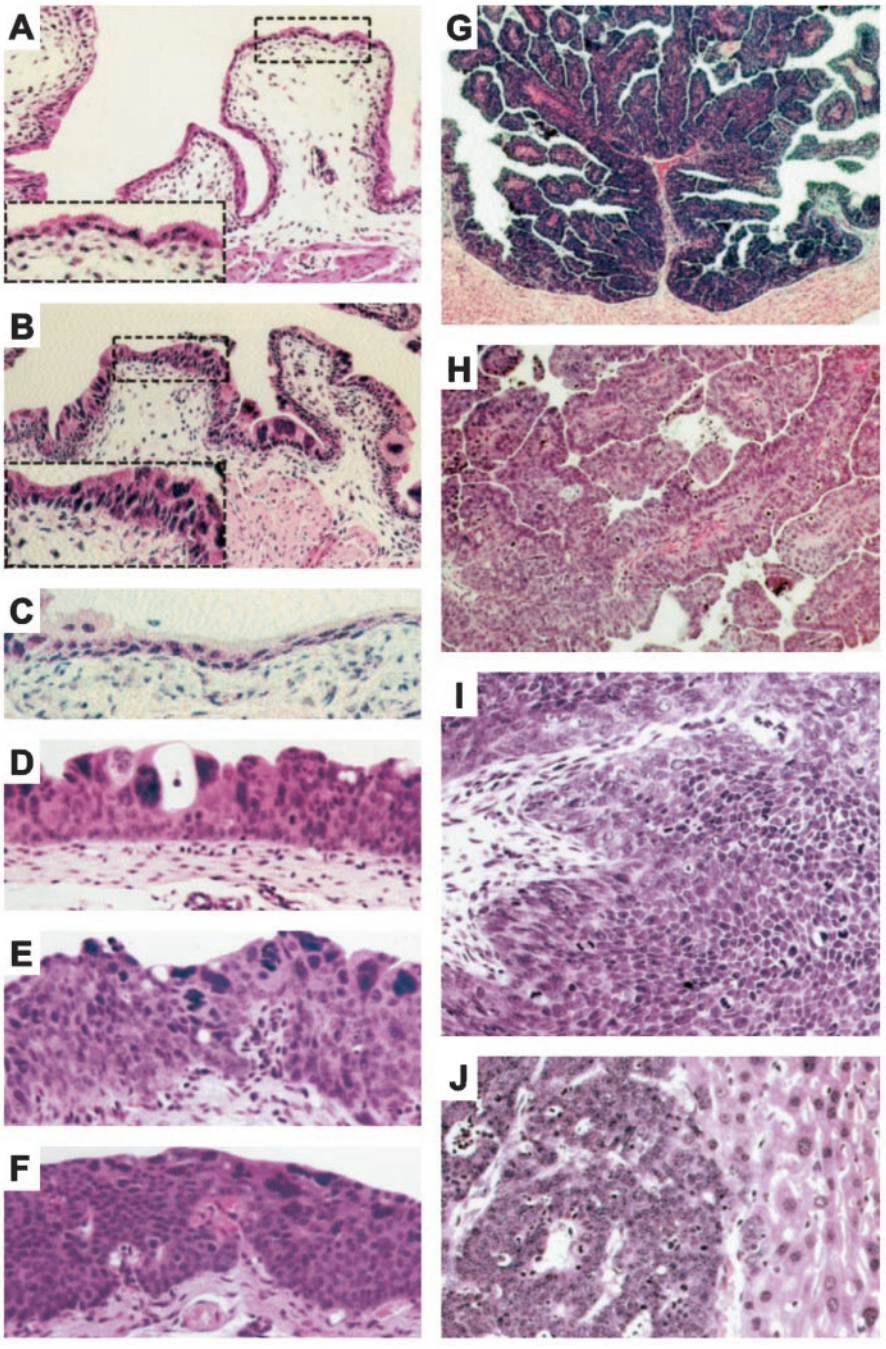

Fig. 1. Histogenesis of urothelial carcinomas in UPII/SV40T transgenic mice. Urinary bladders of transgenic and nontransgenic mice of differing ages were analyzed by paraffin section and H&E staining. *A*, a nontransgenic, newborn mouse showing mucosal folds (attributable to bladder contraction) lined with a very thin urothelium. Under higher magnification (*inset*), urothelium contains two to three layers of cells that are small in size and highly polarized. *B*, a newborn, transgenic mouse showing dysplastic urothelium with increased cell size, nuclear pleomorphism, and hyperchromatism (*inset*). *C*, a 10-month-old nontransgenic mouse showing normal urothelium. *D–F*, three transgenic mice (*D*, 2 months; *E*, 3 months; and *F*, 5 months) showing thickened urothelia with profound nuclear atypia, crowding, high nuclear/cytoplasmic ratio, frequent mitotic figures, and loss of cellular polarity, features resembling human CIS. *G*, a 10-month-old transgenic mouse showing high-grade, superficial papillary tumor of the bladder. *H*, a high-powered view of *G*. *I*, a 15-month-old transgenic mouse showing a poorly differentiated urothelial carcinoma. *J*, liver metastasis of the urothelial carcinoma shown in *I*. Magnifications are ×100 for panels *A*, *B*, *D–F*; ×200 for *C*, *I*, *J*, *insets* of *A* and *B*; ×150 for *H*; and ×50 for *G*.

mice, there was insufficient material for the CGH analysis of this particular urothelial state. Most of the CIS yielded sufficient amounts of genomic DNA for direct probe labeling, except for two tumors (cases 7 and 10) where DOP-PCR was performed for DNA amplification (Table 2). Overall, gains and losses occurred only on a few chromosomes (Fig. 2). The most frequent alteration was the loss of material on chromosome 11, which occurred in 9 of 11 CIS and 2 of 3 high-grade, superficial papillary tumors (Fig. 2; Table 2). Gains were found on chromosome 4 in 6 tumors (43%). Other alterations with two occurrences were losses on chromosomes 7 and 12. These results indicate that the loss of chromosome 11 is a highly selective event in mouse bladder CIS.

Table 1 *Progression of urothelial carcinomas in low-copy-numbered, UPII/SV40T transgenic mice*

	<1 M (n = 21)	1–5 M (n = 20)	6–10 M (n = 24)	11–16 M (n = 12)
Dysplasia	19	0	0	0
CIS	2	20	21	4
HG papillary	0	0	3	8
Muscle invasive	0	0	0	2[a]
Metastatic	0	0	0	2[a]

[a] These two tumors exhibited both invasion and metastases and were also of high-grade, papillary in appearance.

Allelic Loss of p53 Gene by Southern Blotting. Because mouse chromosome 11 is syntenic to human chromosome 17, which harbors the p53 gene, we examined by Southern blotting whether there was a direct correlation between the chromosome 11 loss and p53 gene loss in bladder CIS. This was accomplished by comparing the intensity of p53 pseudogene with that of p53 functional gene. In wild-type mice,

Table 2 *CGH and Southern blot analyses of urothelial carcinomas in UPII/SV40T transgenic mice*

Animal no.	Sex	Age (mo)	Urothelial phenotype	Chromosome gains	Chromosome losses	p53 gene status
1	F	5	CIS			N/D[a]
2	F	5	CIS		11	N/D
3	F	5	CIS	2F2-2H3, 4	9, 10, 11, 12, 16	N/D
4	M	5	CIS		11	N/D
5	M	5	CIS	4[b], 14[b]	11[b]	N/D
6	M	5	CIS		11	+/−
7[c]	M	5	CIS		11	N/D
8	M	5	CIS	4, 14[b]	11	N/D
9	M	5	CIS	4	7, 11	+/−
10[c]	M	5	CIS		7, 11	N/D
11	M	7	CIS	4 (band4C5-qter)	11	N/D
12	F	13	G2-3 Tumor	1[b], 14[b]	10[b], 11	+/−
13	M	13	G2-3 Tumor	3F3-qter, 9F1-qter, 17D-E4	11, 12	+/−
14	F	13	G2-3 Tumor	4		N/D
15	F	5	CIS	N/D	N/D	+/−
16	M	5	CIS	N/D	N/D	+/−
17	M	5	CIS	N/D	N/D	+/−
18	M	7	CIS	N/D	N/D	+/−
19	F	7	CIS	N/D	N/D	+/−
20	M	7	CIS	N/D	N/D	+/−
21	F	8	CIS	N/D	N/D	+/−
22	F	8	CIS	N/D	N/D	+/−
23	F	9	CIS	N/D	N/D	+/−
24	F	4	CIS	N/D	N/D	+/+
25	F	4	CIS	N/D	N/D	+/−

[a] N/D, not determined; CGH was not done on cases 15–25.
[b] Deviation from the baseline not as great as those considered unequivocal.
[c] DOP-PCR.

this ratio was consistently 1:2 (Fig. 3, *Lanes 1* and *4*). However, the ratio was reduced to 1:1 in 2 bladder CIS and 2 high-grade, superficial papillary tumors that showed by CGH allelic loss of chromosome 11 (Table 2), thus providing independent verification of the chromosomal loss. Of the 11 new CIS examined, 10 samples showed such an allelic loss of p53 gene (Fig. 3, *Lanes 5–8* showing 4 representative CIS; Table 2). These results, combined with those obtained from CGH, established the loss of p53-bearing chromosome as a major genetic event in the bladder CIS of the UPII/SV40T transgenic mice.

Expression of Cell Cycle Regulators. To examine the potential alterations in the expression of several key cell cycle regulators in bladder CIS, we performed Western blotting and immunohistochemical staining. Compared with normal urothelium where there was very little p53 protein, all 5 cases of bladder CIS had markedly elevated p53 (Fig. 4, *A* and *B*). This was consistent with the fact that SV40 large T antigen can stabilize p53 protein rendering it nonfunctional. A slight increase was also found with pRb, possibly because of a similar SV40T-mediated stabilization mechanism. Mdm-2, a major downstream target of p53 (19), was greatly diminished, strongly suggesting that p53 was functionally inactivated by the SV40T antigen (Fig. 4*A*). However, the level of p21, also a target of p53 (19), was unaltered (Fig. 4, *A* and *B*), raising the interesting possibility that p21 expression can be regulated by p53-independent mechanisms (see "Discussion"). Consistent with increased cellular proliferation, there was a significant elevation of phosphorylated MAPK level. The fact that most bladder CIS had lost one p53 gene allele, that the p53 protein made by the remaining allele can be functionally inactivated by the SV40T antigen, and that Mdm-2 was dramatically reduced, strongly suggests that p53 was nonfunctional in bladder CIS.

Fig. 3. Allelic loss of p53 gene in bladder CIS of the UPII/SV40T transgenic mice as detected by Southern blot analysis. Genomic DNA from 4 control mice (*Lanes 1–4*) and 4 representative CIS of the transgenic mice (*Lanes 5–8*) were hybridized with a mouse p53 cDNA probe. In wild-type mice (*Lanes 1* and *4*), the intensity of p53 pseudogene (10 kb) to that of p53 functional gene (5 kb) was ~1:2. In heterozygous p53 knockout mice (*Lane 3*), the intensity of the pseudogene to the mutant allele (7 kb) and to the functional gene was 1:1:1. In homozygous p53 knockout mice (*Lane 2*), the intensity of the pseudogene to the mutant allele was 1:2. Note that in all four CIS shown (*Lanes 5–8*), the ratio of the pseudogene to the functional gene was 1:1, indicating allelic loss of the p53 functional gene.

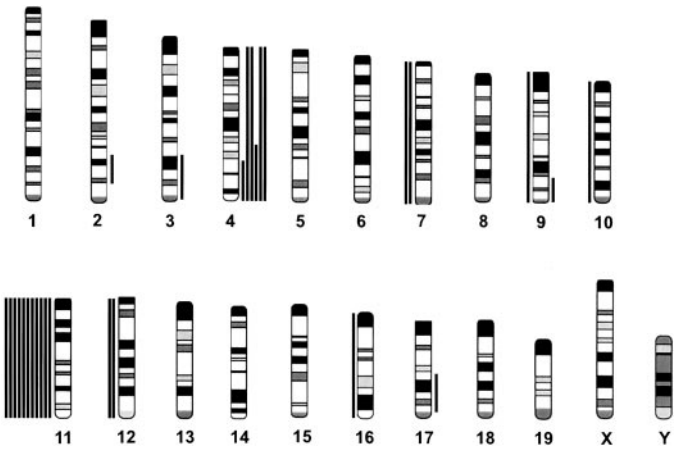

Fig. 2. Compilation of chromosomal alterations in 14 urothelial carcinomas of UPII/SV40T transgenic mice as detected by CGH. *Bars* on the left of chromosome ideograms denote losses and those on the right gains. Note the highly selective loss of material on chromosome 11 and gains on chromosome 4.

DISCUSSION

Stepwise Progression of Urothelial Carcinomas in UPII-SV40T Transgenic Mice. By dissecting urinary bladders from transgenic mice of increasing ages, we were able to track the sequence of bladder

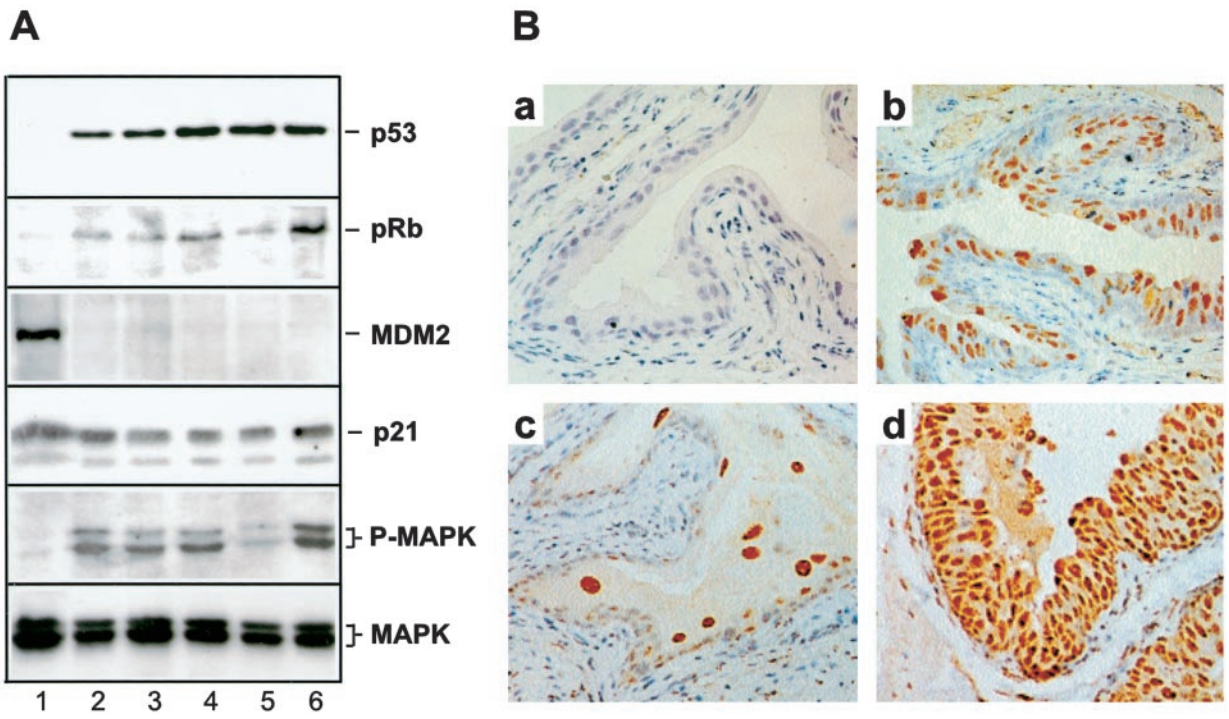

Fig. 4. Expression of cell cycle regulators in the bladder CIS of the UPII/SV40T transgenic mice. *A*, Western blotting analysis. Total urothelial proteins from normal mice (*Lane 1*) and 5 transgenic mice exhibiting bladder CIS (*Lanes 2–6*) were resolved by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with antibodies against p53, pRb, Mdm-2, p21, and phosphorylated MAPK. An antibody against unphosphorylated MAPK was used as a loading control. Note the increased levels of p53 and pRb (attributable to SV40T-mediated stabilization), the drastically decreased level of Mdm-2, and unchanged level of p21 in bladder CIS. Also note the increased level of phosphorylated MAPK in CIS. *B*, immunohistochemical staining. Paraffin sections of normal urothelia (*a* and *c*) and CIS lesions of the transgenic mice (*b* and *d*) were stained with antibodies against p53 (*a* and *b*) and p21 (*c* and *d*). Note the strong staining of both p53 and p21 in bladder CIS. All panels are of the same magnification (×200).

tumor development from dysplasia in newborn mice to CIS in adult mice and then to high-grade, noninvasive papillary tumors in old mice (Fig. 1; Table 1). This analysis established several important points. First, the majority (41 of 44; 93%) of the CIS did not progress immediately (Table 1), but instead they remained as such for ~10 months, which is close to half of a mouse's life span. These CIS lesions may therefore be equivalent to certain primary CIS in humans, where invasion is an infrequent event (7). Second, when CIS did progress in aged mice, the majority (8 of 12; 67%) of them became high-grade, noninvasive, papillary tumors (Fig. 1; Table 1). Interestingly, this type of the tumor is also found in humans and is often graded as pTaG2 and pTaG3 tumors (20, 21). They account for ~3% of all bladder tumors, and their histological origin was uncertain. Our current study suggests that at least some of these tumors may be derived from flat CIS. From a mechanistic standpoint, the progression of CIS to high-grade papillary tumors may represent a focal expansion of cells in the CIS lesions toward the bladder lumen because of a gain of growth potential. Indeed, these tumors were observed at much younger ages in our recent bi-transgenic mice expressing both SV40T and EGFR (22). EGFR overexpression therefore significantly shortened the latency of high-grade, papillary tumors; but it did not trigger invasion, suggesting that enhanced growth does not directly lead to CIS invasion. Third, although none of the mice harboring CIS had metastasis, 2 of 10 mice that had high-grade papillary tumors of the bladder developed distant metastases (Fig. 1; Table 1). Taken together, these results strongly suggest that CIS is an important precursor of high-grade, papillary tumors, the latter of which carries an increased risk of progressing to invasive and metastatic disease.

Highly Selective Loss of a p53-bearing Chromosome 11 in CIS. The most striking genetic alteration of CIS in our transgenic mice is the loss of a p53-bearing mouse chromosome 11. Collectively, 19 of 22 (86%) of the cases examined (11 by CGH and 11 by Southern blotting) showed such an allelic loss (Figs. 2 and 3; Table 2). In contrast, very few other chromosomes had any alterations by CGH, indicating that the loss of a p53-bearing chromosome 11 is a highly selective and early event in mouse CIS. In human CIS, although the loss of chromosome 17, the equivalence of mouse chromosome 11, is also highly prevalent, it is often accompanied by a number of other chromosomal deletions as revealed by partial allelotypic analyses (9–11, 23). A global search of chromosomal imbalances by CGH, which has yet to be done with human CIS, will likely reveal additional chromosomal abnormalities. Our current results suggest other accompanying chromosomal imbalances, albeit also in high frequencies in humans, might represent secondary events that are not relevant to the genesis of CIS.

As mentioned, most mouse CIS that harbored p53 deletions neither invade nor regress for an extended period of time (Table 1). This raises the interesting possibility that p53 dysfunction plays an important role in tumor maintenance. It is known that p53 functions by arresting cell growth and inducing apoptosis (24–27). Compromised p53 function can therefore lead to increased cell growth and reduced apoptosis, both of which are advantageous to tumor maintenance. Our results also suggest that the loss of p53 function alone is insufficient to provoke invasion. As recently suggested, tumor invasion is a complicated process involving a number of genetic and epigenetic factors, including the dysfunction of other tumor suppressor genes, activation of oncogenes, overexpression of vascular growth factor and matrix degrading enzymes, and down-regulation of cell adhesion molecules (5, 28–34). p53 may not be the sole rate-limiting step in CIS invasion, but its dysfunction certainly can lead to genome instability, and as a result, predispose CIS to additional genetic alterations, some of which are required for invasion.

Because it is well known that the oncogenic effect of SV40T antigen primarily depends on its binding and inactivation of p53 and

pRb tumor suppressor proteins (35), it is puzzling why there is a need for the mouse bladder CIS to undergo an allelic loss of p53 gene (Figs. 2 and 3; Table 2). A possible explanation is that the transgenic mice under study harbored low transgene copies and expressed low levels of SV40T antigen, which was insufficient to completely inactivate the p53 protein. Losing a p53 gene allele would additionally reduce the p53 gene dosage and hence its protein product. A similar situation frequently occurs in humans where loss of one p53 gene allele first takes place followed by loss-of-function mutation(s) in the second allele (36–39). In this regard, naturally occurring human CIS and mouse bladder CIS induced by SV40T antigen (Fig. 1) share mechanistic features with respect to p53 gene loss. An alternative explanation is that mouse chromosome 11 may harbor other tumor suppressor genes, the loss of which also benefits tumor maintenance. Indeed, in addition to p53 gene, this chromosome contains other known tumor suppressor genes such as BRCA1 and NF1 (40, 41). The role of these genes in bladder tumorigenesis has yet to be investigated.

Besides chromosomal 11 loss, the only other significant alteration was the gain on chromosomal 4, occurring in 45% (5 of 11) of mouse bladder CIS cases (Fig. 2; Table 2). Human-mouse chromosomal homology map shows that the bulk of chromosomal 4 is syntenic to human chromosomes 1 and 9. The significance of the gains in these regions requires additional study.

It is of interest to note that CIS of the mouse bladder induced by chemical carcinogen such as *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, also harbors p53 and chromosome 4 abnormalities, although those involving chromosome 4 are mainly deletions (42). Invasive tumors are more frequently observed in these chemical carcinogenesis models than in our SV40 T transgenic mice, possibly reflecting the more wide-reaching mutagenic effects of the chemical carcinogens (42, 43).

Independent Regulation of Mdm-2 and p21. We hypothesized that the loss of one p53 gene allele, combined with the inactivation of p53 protein of the remaining allele by SV40T antigen, would render p53 protein completely nonfunctional in mouse CIS. To test this hypothesis, we examined the protein levels of two key p53-regulated genes, Mdm-2 and p21. Mdm-2 was significantly diminished in all mouse tumors compared with normal urothelium, indicating that the p53 regulation of Mdm-2 is compromised. However, the level of p21 remained unchanged as shown by both Western blotting and immunohistochemical staining (Fig. 4), strongly suggesting that p21 can be regulated by other genes. Some of these upstream regulators such as p73 and KLF6 have already been identified and characterized (44, 45). p21 is an important negative cell cycle regulator, and its loss is frequently associated with human bladder tumor progression (19, 46). It would be interesting to see whether the loss of p21 in double transgenic mice expressing SV40T but lacking p21 would provoke CIS invasion.

Progression Pathways of Bladder Cancer: Lessons from Transgenic Mice. The most striking result from our recent transgenic models is that different genetic alterations introduced into the *in vivo* urothelium are capable of inducing phenotypically different tumors. Thus, urothelial overexpression of either activated Ha-ras or EGFR can elicit urothelial hyperplasia (Fig. 5; Refs. 12, 22). The persistent expression of activated Ha-ras, but not EGFR, can result in the formation of low-grade, superficial papillary tumors (12, 22). These observations clearly define the role of Ha-ras signaling in causing low-grade, superficial papillary urothelial tumors, which account for 70–80% of all human bladder cancers (47, 48). Although the mutation rate of Ha-ras in human bladder cancer remains somewhat controversial, the Ha-ras signaling pathway, which mediates the cellular responses to many oncogenes and growth factors, may indeed be activated in a high proportion of human bladder cancer (49). Throughout the lifespan of the transgenic mice, the ras-induced papillary tumors remain low-grade and noninvasive, indicating that these tumors lack the biological potential to progress (Fig. 5). In stark contrast, the SV40T antigen, which selectively inactivates p53 and pRb, induces exclusively high-grade bladder cancers in transgenic mice. The pace of tumor progression in the SV40T transgenic mice depends, however, on the level of transgene expression. Low-level expression elicits urothelial dysplasia and CIS, the latter of which has a complete loss of p53 function but can remain noninvasive for an extended period of time. The majority of CIS eventually progresses to become high-grade, superficial papillary tumors, a process that can be significantly accelerated by growth-promoting signals such as the overexpression of EGFR. High-level expression of SV40T antigen induces CIS, which quickly advances to invasive and metastatic tumors, strongly suggesting that the complete abolishment of p53 as well as pRb function is key to the aggressive behavior of bladder cancer (Fig. 5; Refs. 13, 37, 38, 50–52). These transgenic studies therefore strongly support and extend the pathway concept derived from human studies (1, 9, 10, 23, 38, 53). The fact that bladder cancers in these transgenic mice harboring well-defined genetic defects evolve in a highly stochastic fashion also raises the important question regarding the possible existence of other genetic and epigenetic factors required for invasion and progression. Additional studies combining transgenic and knockout mice should shed new light on how oncogenes and tumor suppressor genes cooperate in driving bladder cancer progression.

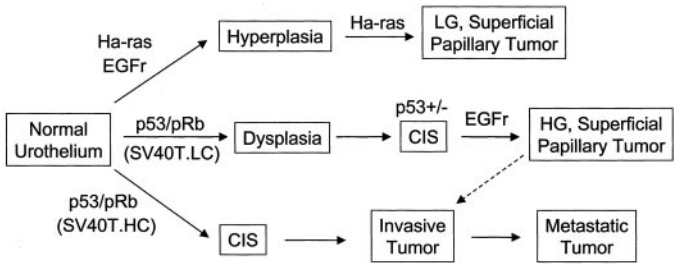

Fig. 5. Phenotypic and molecular pathways of bladder tumorigenesis as revealed by transgenic mouse studies. Analyses on several transgenic models indicate that bladder cancer develops via multiple stages and along distinctive pathways. *Top*, Ha-ras activation or EGFR overexpression induces urothelial hyperplasia followed by low-grade (LG), superficial papillary bladder tumors. These findings strongly suggest the role of Ha-ras signaling pathway in the low-grade, superficial papillary pathway of bladder tumorigenesis (12, 22). *Middle*, inactivation of p53 and pRb by the low-level expression of SV40T antigen (SV40T.LC) elicits urothelial dysplasia, which progresses to CIS. CIS persists without progression or regression for a long time period during which there is a highly selective loss of a p53-bearing chromosome (p53+/−). CIS eventually advances to become high-grade (HG), superficial papillary tumors, a process that can be significantly accelerated by the overexpression of EGFR. These results suggest that high-grade, superficial papillary tumor is an important intermediary between CIS and invasive tumors. They also suggest the importance of allelic loss of p53 gene in bladder tumor maintenance and the growth- but not the invasion-enhancing role of EGFR overexpression. *Bottom*, high-level expression of SV40T antigen (SV40T.HC) induces CIS, which quickly advances to invasive and metastatic tumors, strongly suggesting that the complete abolishment of p53 and pRb function is key to the aggressive behavior of bladder cancer (13).

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