Urothelium-specific Expression of an Oncogene in Transgenic Mice Induced the Formation of Carcinoma in Situ and Invasive Transitional Cell Carcinoma

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ABSTRACT

Although many genetic alterations are known to be associated with human transitional cell carcinoma (TCC) of the urinary bladder, relatively little is known about the roles of these molecular defects, singular or in combination, in bladder tumorigenesis. We have developed a transgenic mouse model of bladder tumorigenesis using a 3.6-kb promoter of uroplakin II gene to drive the urothelial-specific expression of oncoproteins. In this study, we demonstrate that transgenic mice bearing a low copy number of SV40T transgene developed bladder carcinoma in situ (CIS), whereas those bearing high copies developed CIS as well as invasive and metastatic TCCs. These results indicate that the SV40T inactivation of p53 and retinoblastoma gene products, defects frequently found in human bladder CIS and invasive TCCs, can cause the aggressive form of TCC. Our results also provide experimental proof that CIS is a precursor of invasive TCCs, thus supporting the concept of two distinct pathways of bladder tumorigenesis (papillary versus CIS/invasive TCC). This transgenic system can be used for the systematic dissection of the roles of individual or combinations of specific molecular events in bladder tumorigenesis.

INTRODUCTION

TCC of the urinary bladder ranks fifth among the most common neoplasms and twelfth among the leading causes of cancer deaths in the United States (1). One of the most important advances in studying bladder TCC is the recognition of two TCC variants (2). The first variant constitutes the majority (80%) of TCCs and is presented as superficial, noninvasive papillary lesions. This variant is often multifocal and tends to recur but has limited potential to progress to muscle invasion (3). The second variant presents as an invasive tumor at diagnosis and has a high risk of progressing to distant metastases, thus posing a major challenge in clinical management (4). The interrelationship between these two variants has been studied extensively. It has been suggested that some invasive TCCs can arise from high-grade superficial papillary TCCs (5), although this can at best account for only 10–25% of the invasive tumors. It has also been suggested that invasive TCCs can arise de novo, because some patients have no prior history of superficial lesions (6, 7). Lastly, longitudinal studies indicate that up to 60% of CIS will progress to invasive TCCs, suggesting that the majority of invasive TCCs are cis derived (8, 9). These data strongly suggest that bladder tumors can occur via at least two pathways that give rise to papillary TCC and CIS/invasive TCCs (10).

Recent studies indicate that the two TCC variants are characterized by different genetic alterations. Spruck et al. (11) observed the loss of heterozygosity of chromosome 9 in 34% of the superficial papillary TCCs but only in 12% of the CIS. In contrast, p53 mutations occur in 51 and 65% of the CIS and invasive TCCs, respectively, but only in 3% of the papillary TCCs. Rosin et al. (12) and Wagner et al. (13) also found that CIS and invasive TCCs harbor similar genetic changes, which are not shared by papillary TCCs. Although these results strongly suggest that CIS is closely related to the invasive TCCs, there is thus far no experimental system in which this precursor-product relationship can be studied. Moreover, although many genetic defects are prevalent in human bladder TCCs, including the inactivation of tumor suppressor genes p53 and Rb, as well as the activation and/or overexpression of H-ras, erbB-2, c-myc, and epidermal growth factor receptor (14–20), the precise roles of these genetic alterations in bladder tumorigenesis remain unclear.

The transgenic mouse provides an ideal system for dissecting the roles of these molecular events, individually or in combination, in bladder tumorigenesis. A prerequisite of this approach is the availability of a promoter that can drive individual oncoproteins to express in a urothelium-specific fashion. Toward this goal, we have shown recently that a 3.6-kb 5′-upstream sequence of mouse uroplakin II gene can drive a LacZ reporter gene and a human growth hormone gene to express at a high level in the urothelium of transgenic mice (21, 22). In this report, we show that this promoter can be used to drive the urothelium-specific expression of SV40T antigen. SV40T is an oncoprotein that can inactivate p53 and retinoblastoma protein, two important tumor suppressor gene products that are frequently mutated in human bladder TCCs (23, 24). We found that the urothelial expression of SV40T in transgenic mouse induced transitional cell carcinomas that bear strong resemblance, not only in phenotypes but also in the mode of progression, with human TCCs. The establishment of this transgenic model of bladder tumorigenesis provides a tool for a systematic dissection of the molecular pathways of bladder tumor formation and for testing preventive and therapeutic strategies.

MATERIALS AND METHODS

Generation of Transgenic mice. The uroplakin II-SV40T chimeric gene was constructed by fusing marine uroplakin II gene promoter with the coding sequences of SV40 large T oncogene. A 3.6-kb KpnI-BamHI fragment containing the UP II promoter was subcloned into pBluescript SK+ (Stratagene, La Jolla, CA; Ref. 25). The transgene was cloned into pBluescript SK+ and probed with a 32 P-labeled, BamHI fragment containing the SV40T antigen (26). For the estimation of transgene copy number, Southern Blotting of Mouse Tail Genomic DNA. Mouse tail DNA was digested with NcoI and probed with a 32 P-labeled, StuI-BamHI (500-bp) fragment from SV40T oncogene. For the estimation of transgene copy number, a 600-bp PsI fragment of UPII promoter was used as a probe to detect endogenous as well as transgenics. The X-ray film of genomic Southern blot was subjected to densitometry for the calculation of the relative amount of transgene by comparing transgene with the endogenous UPII gene.

Histological, Immunohistochemical, and Immunofluorescent Staining. Mouse tissues were fixed in 10% buffered formalin and embedded in paraffin. Three to five-μm sections were stained with H&E and examined microscop-
ically for pathological changes. For the staining of SV40 large T antigen, paraffin sections were digested with 0.25% trypsin in 25 mM Tris/HCl (pH 7.8) at 37°C for 15 min and incubated with a rabbit antiserum against large T antigen (courtesy of Douglas Hanahan of University of California, San Francisco, CA; Ref. 27). After washing in PBS, the sections were incubated with a goat anti-rabbit antibody conjugated with horseradish peroxidase and developed in a diamine benzidine/H$_2$O$_2$ solution. For retrieval of uroplakin antigens, paraffin sections were microwaved in citrate buffer (pH 6.0) for 15 min and then stained with a rabbit pan-uroplakin antibody (28), followed by a peroxidase-conjugated secondary antibody. For immunofluorescent staining, tissue blocks were fixed in Zamboni’s solution (2% paraformaldehyde plus 15% picric acid) and embedded in OCT compound. Five-micrometer-thick cryosections were then stained by indirect immunofluorescence (28).

### RESULTS

**Generation of Transgenic Mice Harboring SV40 Large T Oncogene.** We have shown previously that UPII protein is detectable only in the superficially located, terminally differentiated umbrella cells of bovine urothelium (Fig. 1c; Ref. 29). However, in mouse urothelium, UPII is expressed much earlier in suprabasal cells; even the basal cells are weakly stained by antibodies to uroplakins, suggesting a much earlier onset of uroplakin expression (Fig. 1b). Although the biological significance of such a species-dependent mode of uroplakin expression is unclear, the fact that UPII promoter can function even in the relatively undifferentiated mouse urothelial suprabasal and, to some extent, basal cells, that are more likely to be the target of oncogenic actions, greatly enhances the usefulness of the promoter for studying bladder tumorigenesis.

We have thus constructed a chimeric gene composed of a 3.6-kb mouse UPII promoter and a 2.7-kb SV40T oncogene (Fig. 2a) and used it to generate transgenic mice. Southern blotting of mouse tail DNAs showed that 4 of 30 live-born mice had incorporated the chimeric gene (Fig. 2b). The transgenes of the four founder mice were inserted into independent sites, all in tandem repeats (Fig. 2b). Founders 9 and 19 harbored two copies of the transgene per haploid genome, whereas founders 2 and 29 harbored 10 and 6 copies, respectively. Southern blotting of F1 mouse DNAs showed that founders 9, 19, and 29 transmitted the transgene to their progenies (Fig. 2b).

**Transgenic Mice Harboring a Low Copy Number of SV40T Transgene Developed Bladder Carcinoma in Situ.** All four transgenic lines showed urothelial abnormalities, with no detectable changes in any other organs. The severity of the urothelial changes varied, depending on the copy number of the transgene (Table 1). Mice harboring only two copies of the transgene (F0.9 and F0.19) appeared outwardly healthy. Histological examination of the bladders from 30 F1 mice, which were 5 to 8 months of age, showed consistently, however, features closely mimicking those of the bladder CIS in humans including moderate-to-severe urothelial atypia with marked nuclear pleomorphism such as giant nuclei and abundant mitotic figures. These anaplastic cells were confined to
the epithelial layers because the basement membrane remained intact (Fig. 3; Table 1).

**Mice Harbor the High Copy Number of the Transgene Developed Invasive Transitional Cell Carcinomas.** The two founder mice (numbers 2 and 29) carrying high copy numbers of transgenes succumbed with bladder cancer at the ages of 3 and 5 months, respectively. The F0.2 mouse (10 copies) developed, in addition to patches of CIS (Fig. 4b), a moderately differentiated TCC that had invaded into the detrusor muscle layer (Fig. 4, a–c; inset; see below). The F0.29 mouse (six copies) developed a palpable abdominal tumor at 5 months of age. A tumor mass of 3-cm in diameter was found to occupy the entire pelvic region and encompassed pelvic organs including the bladder, prostate, seminal vesicles, and rectum (Fig. 4f, inset). The histology of the tumor showed a poorly differentiated TCC composed of dense anaplastic cells with scant cytoplasm (Fig. 4f). Micrometastases were found in the pelvic lymph nodes (Fig. 4g) and lingular lobe of the liver (data not shown).

To determine the time course of TCC formation, we sacrificed the F1 mice of high-copy-numbered line 29 at early ages. We found that all 13 young animals at 1–2 months exhibited only bladder CIS changes. These results, combined with the fact that CIS and invasive TCCs coexisted in the same bladder of line 2 (Fig. 4, a and b), indicate that, in our transgenic mice, the CIS can indeed progress, giving rise to invasive and metastatic TCCs.

**Expression of SV40T and UPII in Mouse TCCs.** As UPII gene expression is known to be differentiation related, we wanted to know whether SV40T expression persisted in the tumors that were in general less differentiated than normal urothelium. Immunohistochemical staining using a rabbit anti-T antibody showed that the nuclei of almost all of the mouse TCC cells were SV40T positive (Fig. 5, a and b). Most of SV40T-positive tumor cells also contained uroplakins, albeit at a lower level than normal urothelial cells (Figs. 1 and 5a); this finding is consistent with our previous observations that many human TCCs are uroplakin positive (30, 31).

**DISCUSSION**

**Urothelium Specificity and Differentiation Dependence of the Mouse UPII Promoter.** For the UPII promoter to be useful for studying bladder tumorigenesis, it needs to satisfy two important criteria: (a) it should be urothelium specific. In an earlier study, we found that the 3.6-kb mouse UPII promoter can drive the LacZ gene to express in transgenic mice not only in urothelium but also in hypothalamus (21, 32). On the basis of the fact that ectopic brain expression of the LacZ gene had been seen in many transgenic systems, we speculated that some intrinsic signals of the LacZ gene per se may be responsible for the brain expression. In a recent study, we found that the UPII promoter-driven expression of human growth hormone gene was more than 100-fold higher in the bladder than in the brain, kidney, and testis (22). Consistent with this, we found in the present study that SV40T was expressed exclusively in urothelium, with no PCR-detectable expression in the brain, which remained histologically normal (data not shown). Collectively, these data strongly suggest that our 3.6-kb mouse UPII promoter is indeed highly urothelium specific.

(b) Given the fact that epithelial tumors are almost certainly derived from the stem cells that reside in the basal cell layer (33), the promoter should be active in the lower urothelial cell layers preferentially including the basal cells. Although in bovine and human urothelia UPII expression should be active in the lower urothelial cell layers preferentially including the basal cells. Although in bovine and human urothelia UPII expression is distinctly limited to the superficially located, terminally differentiated umbrella cells (Fig. 1c; 30, 31), in mouse urothelium its expression begins much earlier in the suprabasal layer (Fig. 1b). In fact, a small
amount of uroplakins can be detected even in the basal cells of mouse urothelium (Fig. 1b). Although we do not yet understand the physiological significance of this species-dependent difference in the onset of uroplakin expression, our finding clearly establishes the suitability of the mouse UPII promoter for bladder tumorigenesis studies.

In addition to being useful for directing the urothelial expression of oncogenes, the UPII promoter can also be used to drive the urothelial expression of the Cre gene that can be used to affect the urothelium-specific knockout of tumor suppressor genes such as p53 and Rb (34). The availability of a panel of transgenic and knockout mice that overexpress individual oncogenes or harbor inactivated tumor suppressor genes, coupled with breeding studies, should enable us to study rather systematically the contribution of such genetic defects in bladder tumor formation.

Pathways of Bladder Tumorigenesis. An important finding in our study is that all of our transgenic mice that express the SV40T in their urothelia developed high-grade CIS as well as invasive and metastatic TCCs, without forming any papillary tumors. This finding has two major implications:

(a) It indicates that the inactivation of p53 and Rb tumor suppressor proteins by SV40T antigen can induce the formation of the invasive variant of TCCs but cannot induce the superficial variant of TCCs. Our finding is, therefore, quite consistent with existing human data indicating that p53 and Rb mutations are far more prevalent in invasive TCCs than in superficial TCCs (11–13).

(b) Our findings provide strong experimental support to the concept that CIS is a precursor of invasive TCCs. The invasive potential of human CIS has long been recognized since the first description of the lesion (35). Longitudinal studies show that up to 60% of the patients with CIS develop invasive disease within 5 years and 80% within 10 years (36). In patients presenting with invasive TCCs, CIS is a frequent cofinding. Recent genetic studies further suggest that CIS has already acquired several genetic changes commonly found in invasive bladder tumors (11–13). Our transgenic study indicates that: a well-defined genetic change, i.e., the urothelial expression of SV40T, resulted in the formation of both CIS and invasive TCCs in all transgenic pedigrees; that CIS and invasive TCCs coexisted in the same bladder; and that CIS preceded invasive TCCs in high-copy-numbered transgenic mice (Table 1). Our data thus provide strong experimental evidence supporting the concept that CIS can progress to become invasive TCCs and suggest that the transgenic mouse model as described here can be suitable for further, detailed analysis of this progression.

Although CIS clearly has a potential to invade, not all CIS lesions progress to invasive TCCs in humans (9). The same holds true in our transgenic mouse model, because low-copy-numbered transgenic mice remain invasion-free for up to 8 months, suggesting that a residual amount of p53 and Rb might be present in these mice. The overexpression of SV40T antigen, as occurring in the high-copy-numbered animals, seems sufficient to cause the CIS to progress and acquire the invasive TCC phenotype, even in young animals <5 months in age. This suggests...
that the complete inactivation of both p53 and Rb is critical for CIS progression. It has been shown in humans that the loss of both p53 and Rb genes is a stronger indicator for TCC progression than the loss of one gene alone (37–39). Additional genetic and/or epigenetic events may also be involved in TCC progression; these may include the overexpression of certain growth-promoting signals including H-ras, epidermal growth factor receptors, vascular endothelial growth factor, or the inactivation of other tumor suppressor genes (10, 40–45).

Our present SV40T transgenic model does not address the cause and mode of progression of the superficial variant of TCCs, because none of the four transgenic pedigrees studied manifested any well-differentiated, superficial, papillary TCCs. Studies of human papillary TCCs have shown a strong correlation between chromosome 9p21 deletion and the superficial TCCs (42). Recent studies indicate that this region contains a p16INK4a tumor suppressor gene (46) that encodes a cell cycle inhibitor. Its ablation in mice resulted in the development of spontaneous soft-tissue tumors at an early age (47). This gene was found to be inactivated in some human tumors including melanoma and pancreatic carcinoma (48) and in 18% of TCC cases (49). Although p16 deletion can be found in some of the invasive TCCs, it is mainly associated with superficial papillary TCCs (49, 50). The fact that superficial TCCs are characterized by genetic alterations such as p16 deletion, which are distinct from those associated with aggressive TCCs (e.g., p53 and Rb), strongly suggests that different molecular pathways give rise to superficial TCCs versus the CIS/invasive TCCs (10–13, 15, 19, 40–42, 49). Additional transgenic and tissue-specific knockout studies using the urothelium-specific promoter as described here will allow further dissection of these different molecular pathways.

Uroplakin Expression in Mouse and Human TCCs. We have shown previously that uroplakins are differentiation-dependent markers in normal human urothelium being detected mainly, as in bovine urothelium, in the superficial umbrella cells (30, 31). A survey of human TCCs showed that 40% of the invasive TCC cases contained scattered uroplakin-positive cells (30, 31). We have recently improved the sensitivity and reliability of this assay using the RT-PCR technique, which now can detect UPII mRNA in 100% of the cases of advanced, muscle-invasive TCCs. The same technique enabled us to detect a single circulating bladder cancer cells in 5 ml of peripheral blood (51). These results have clearly established the usefulness of uroplakin as a marker for the positive identification of bladder-derived metastatic and poorly differentiated tumors.

We found that the invasive, high-grade mouse TCCs of line 2 (F0.2),
which showed strong nuclear SV40T staining, remained to be almost uniformly uroplakin positive, suggesting that the promoters of both the endogenous uroplakin gene and the UPII-SV40T transgene must be both active, even in these advanced TCC cells (Fig. 5, a and Sa′). That mouse uroplakin gene is less suppressed in TCC than the human situation may be explained by the fact that mouse uroplakin genes are less differentiation dependent than the human ones (Fig. 1; Refs. 30 and 31). The fact that the UPII promoter apparently remained active, even in relatively advanced mouse bladder TCCs, is significant, because it translated into continued presence of the oncogenic gene product during the initial stages of malignant transformation. Interestingly, the poorly differentiated anaplastic tumors of the F0.29 founder mouse were SV40T positive but uroplakin negative (Fig. 5, b and Sb′), possibly reflecting the particularly advanced stage of such TCCs. The fact that these cells remained SV40T positive could be explained if the SV40T protein has a longer half-life than uroplakins, which may not be able to process and assemble properly in poorly differentiated bladder tumor cells.

Concluding Remarks. The transgenic mouse system that we describe here provides the first in vivo platform for dissecting the roles of specific genetic alterations that have been identified in human TCCs on the growth, differentiation, and transformation of urothelium; for studying different pathways of bladder cancer formation; and for testing novel preventive and therapeutic strategies.

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