

Accelerated Induction of Bladder Cancer in *Patched* Heterozygous Mutant Mice

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Abstract

The *PATCHED* (*PTC*) gene is recognized as a tumor suppressor in basal cell carcinoma. Mapping of a minimal region of deletion at 9q22.3 and observation of a decreased *PTC* expression in superficial papillary bladder tumors led us to hypothesize that it could also be involved in this cancer. To further investigate this hypothesis, we submitted *Ptc*^{+/-} heterozygous mutant mice and their wild-type littermates to chemical carcinogenesis by adding *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine to their drinking water. Preneoplastic and neoplastic changes were observed significantly earlier in the *Ptc*^{+/-} than in the wild-type mice. Our data support the hypothesis of *Ptc* acting as a tumor suppressor gene in bladder cancer.

Introduction

The presence of tumor suppressor genes (TSGs) on human chromosome 9, implicated in the pathogenesis of bladder cancer, has been suggested by various groups (1). In an attempt to identify candidate chromosome 9 TSGs, we conducted a mapping of losses of heterozygosity of microsatellite markers in initial superficial papillary bladder tumors (Ta/T1; Refs. 2, 3). This mapping led us to define a 0.5 cM minimal region of deletion at 9q22.3 encompassing the *PATCHED* (*PTC*) gene. A marker within this gene was deleted in 46% of tumors. Moreover, semiquantitative reverse transcription-PCR mRNA expression analysis showed a significant decrease in the expression of *PTC* in tumors with loss of heterozygosity of the *PTC* marker (3). We also observed a significantly increased tumor recurrence rate in patients with tumors having loss of heterozygosity at the 9q22 locus (4). These observations led us to hypothesize that *PTC* could be a TSG involved in bladder cancer. *PTC* has already been recognized as a TSG in basal cell carcinomas (BCCs; Ref. 5) and in medulloblastomas (6). Although *Ptc* homozygous mutant mice die at early stages of embryogenesis, *Ptc* heterozygous mutant mice, in addition to presenting a small proportion of developmental abnormalities, also spontaneously develop tumors such as medulloblastomas and, more rarely, soft tissue tumors (7). However, these mice have not been reported to spontaneously develop BCCs unless exposed to UV or ionizing radiation (8). As is the case for BCCs, the etiology of transitional cell carcinomas (TCCs) has been associated with environmental factors such as industrial carcinogens and cigarette smoking. Superficial papillary bladder tumors and BCCs share similar features of local growth with limited invasiveness and low propensity to metastasize. The genotoxic carcinogen *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) has long been used to induce bladder cancer in rodents (9). To test for a possible involvement of the *PTC* gene in bladder carcinogenesis, we

submitted *Ptc* heterozygous mutant mice to chemical carcinogenesis using BBN. Our data show an accelerated development of carcinogen-induced bladder cancer in mutant mice compared with their wild-type (wt) littermates.

Materials and Methods

Mice. *Ptc* heterozygous mutant mice, from the C57BL/6;129 mixed strain, were purchased from The Jackson Laboratory (Bar Harbor, ME). These mice carry a deletion encompassing both exon 1 and exon 2 of the *Ptc* gene (7). Genotyping was performed by Southern blotting on *EcoRV*-digested tail DNA, using a *Ptc* intron 2 1000-bp *SacI* fragment from a genomic *Ptc* clone (obtained from Dr. Matthew P. Scott, Stanford University, Stanford, CA) as a probe (7). The animals were housed in plastic cages (three mice/cage) on wood chips bedding, in a controlled-environment room at 24°C ± 2°C and 40–70% humidity, with a 12-h light/dark cycle. All of the animals received food and demineralized water *ad libitum*.

Exposure to Carcinogen. Six-to-8-week-old male *Ptc*^{+/-} mice (*n* = 92) and their wt littermates (*n* = 128) were given drinking water containing 0.025% (v/v) BBN (TCI America, Portland, OR), whereas control mice (31 *Ptc*^{+/-} and 32 wt) received only water. The number of experimental *Ptc*^{+/-} mice was lower than the number of wt mice because we had to euthanize ~13% of *Ptc*^{+/-} mice who developed brain tumors at an early age (<6 weeks). The BBN solution was prepared freshly every week and given in brown bottles replaced three times a week. Randomly selected mice were sacrificed at weeks 4 (12 wt, 9 *Ptc*^{+/-}), 8 (13 wt, 12 *Ptc*^{+/-}), 12 (15 wt, 10 *Ptc*^{+/-}), 14 (16 wt, 11 *Ptc*^{+/-}), 16 (20 wt, 17 *Ptc*^{+/-}), 18 (18 wt, 12 *Ptc*^{+/-}), 20 (18 wt, 13 *Ptc*^{+/-}), and 24 (16 wt, 8 *Ptc*^{+/-}) for experimental mice and at weeks 4 (16 wt, 16 *Ptc*^{+/-}) and 24 (16 wt, 15 *Ptc*^{+/-}) for controls. A few mice in both groups (*n* = 7) were euthanized earlier than intended because of poor health status. As an additional control, unexposed mice older than one year (7 *Ptc*^{+/-} and 5 wt) were also sacrificed. In a second experiment, 6-to-8-week-old male *Ptc*^{+/-} (*n* = 5) and wt (*n* = 6) mice were put under BBN for 8 weeks, in exactly the same conditions as for the first experiment. They were housed in a metabolic cage for 24 h at 8-day intervals (four times), to evaluate food and water intake and urine excretion.

Tissue Preparation and Observation. At necropsy, urinary bladders were surgically excised. Approximately one-half of the bladders, randomly selected, were inflated with and fixed in Tissufix-2 (Laboratoire Gilles Chaput, Montr  al, Canada; 48 *Ptc*^{+/-} and 68 wt), and the others were frozen in isopentane in liquid nitrogen and kept at -80°C for subsequent experiments. To make sampling consistent, fixed bladders were sectioned in the mid-sagittal plane, then paraffin embedded. Twelve 5-  m-sections were obtained from the corresponding tissue blocks, and the first and last sections were stained with H&E. Thus, each urinary bladder yielded two separate areas for histological observation, from which the extent of neoplastic involvement of the bladder as a whole was evaluated. The histopathological features of the tissues were recorded by two independent observers (H. H. and S. H.) with subsequent review by a uro-pathologist (E. L.). The histopathological terminology used in this study was based on the International Classification of Rodent Tumors (10). Criteria for describing tumor invasion were similar to those used for human tumors, as described by Dunn *et al.* (11). Tissues were evaluated blindly, the exposure status being unknown to the evaluator.

Immunohistochemistry and the TUNEL Reaction. Immunohistochemical detection of actively dividing cells was performed using an anti-phosphohistone H3 rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) as previously described using antigen retrieval (12). Antibody detection was carried out with the Vectastain Elite kit (Vector Laboratories, Burlingame,

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CA), using biotinylated goat antirabbit IgG. Staining was done with diaminobenzidine (Zymed Laboratories, San Francisco, CA). Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) reaction (13). Fragmented DNA was nick end labeled with biotinylated dUTP and detection was carried out with the Vectastain Elite ABC kit. In both cases, counterstaining was done using Mayer's hematoxylin. Stained nuclei were counted by two independent observers (H. H. and J. G.), and the result was expressed as the percentage of positive nuclei per total cells counted. Approximately 250 nuclei were counted in a minimum of two separate zones for phosphohistone H3. Between 100 and 700 nuclei were counted in five or six separate regions after the TUNEL reaction. Normal urothelium, hyperplastic urothelium, and cancer areas were evaluated separately.

Statistical Analysis. Comparison of proportions of *Ptc*^{+/-} and wt mice with preneoplastic and neoplastic changes was based on the Jonckheere-Terpstra test for ordinal outcome. Comparison of proportions of *Ptc*^{+/-} and wt mice with and without carcinomas was based on the Fisher's exact test. Comparison of proliferation levels based on phosphohistone H3 staining and of the proportion of apoptotic cells based on the TUNEL reaction was carried out using the Student's *t* test.

Results

Histopathological Observations. Pathological analysis of the tissues was carried out on two H&E-stained sections from each of the 48 *Ptc*^{+/-} and 68 wt fixed bladders from experimental mice and from each of the 15 *Ptc*^{+/-} and 17 wt fixed bladders from control mice. Tumors developed in the bladders of *Ptc*^{+/-} and wt mice. Fig. 1 shows H&E staining of normal urothelium (Fig. 1A), hyperplasia (Fig. 1B) and superficial (Fig. 1C) and invasive (Fig. 1D) tumors. However, preneoplastic and neoplastic changes occurred significantly earlier in *Ptc*^{+/-} mice (Table 1; Fig. 2). After

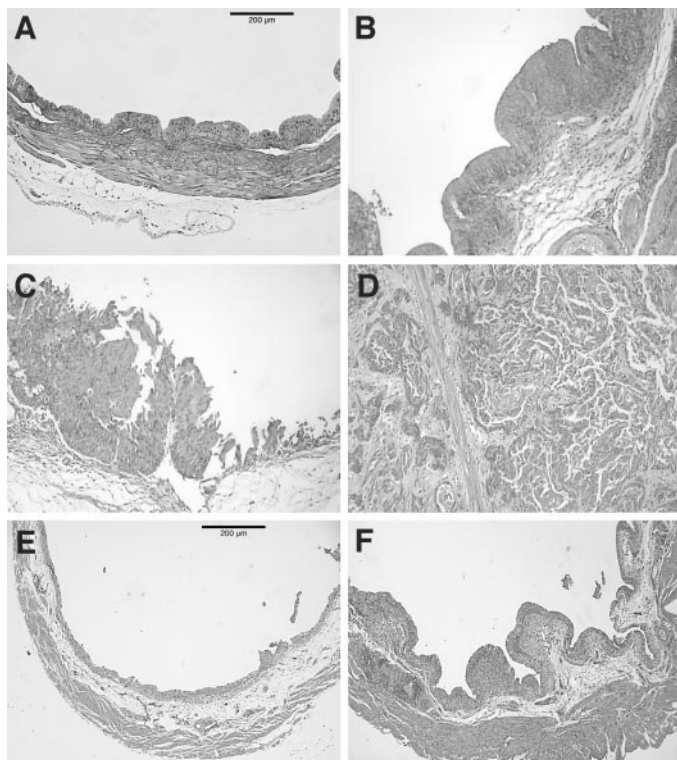


Fig. 1. H&E staining of mouse bladder sections: A, normal bladder from a control wild-type (wt) mouse; B, urothelial hyperplasia in a *Ptc*^{+/-} mouse; C, noninvasive low-grade papillary transitional cell carcinoma from a wt mouse; D, invasive transitional cell carcinoma with squamous differentiation from a wt mouse. (A–D, $\times 20$). E, urothelium from a wt mouse after 8 weeks of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine administration, showing edema. F, urothelium from a *Ptc*^{+/-} mouse after 8 weeks of BBN administration, showing edema accompanied by stromal proliferation. (E–F, $\times 4$).

8 weeks of BBN administration, hyperplasia was observed in the urothelium of seven of seven *Ptc*^{+/-} mice (Fig. 1B) compared with only three of seven wt mice. Multiple foci of atypical hyperplasia were observed in two *Ptc*^{+/-} mice, one of which also presented a unique TCC with squamous differentiation invading the lamina propria. None of these were observed in wt mice. This difference was significant by Jonckheere-Terpstra test ($P = 0.036$). By 12 weeks, almost one-half (2 of 5) of *Ptc*^{+/-} mice had developed multiple TCCs, whereas this proportion was attained only after 16 weeks in wt mice, with 5 of 10 mice presenting multiple TCCs. At that time, all of the *Ptc*^{+/-} mice (11 of 11) had developed TCCs, a highly significant difference when compared with the wt mice (Jonckheere-Terpstra test, $P = 0.009$). The difference between wt and *Ptc*^{+/-} mice for the incidence of carcinomas was also significant using the Fisher's exact test ($P = 0.0124$). In *Ptc*^{+/-} mice, squamous differentiation was observed in 7 of 11 cases and multiple foci in 10 of 11 cases, and all of the tumors were invasive to various degrees. From 16 weeks on, all *Ptc*^{+/-} mice had TCCs and after 20 weeks, all were high-grade invasive TCCs with squamous differentiation. The presence of tumors in all of the wt mice was observed only after 24 weeks of BBN, with 8 of 8 mice presenting high-grade tumors, 7 of 8 being invasive, and 6 of 8 with squamous differentiation (Fig. 1D). Although tumors appeared more rapidly in *Ptc*^{+/-} mice, no difference was observed in the degree of invasiveness between wt and *Ptc*^{+/-}. Invasion progressed from microfoci of cancer cells in the lamina propria, through the muscle and the serosa and up to infiltration into neighboring tissues in a few cases.

After only 4 weeks of treatment, even before the onset of preneoplastic changes, edema was observed in the urothelium of both wt and *Ptc*^{+/-} mice. A marked difference was observed in the extent of the phenomenon, with four of five *Ptc*^{+/-} but only two of six wt mice presenting a high degree of edema at that time. The edema was accompanied by foci of stromal cell proliferation that were seen only in *Ptc*^{+/-} mice (Fig. 1, E and F) and that were still more noticeable after 8 weeks in all 7 of the *Ptc*^{+/-} mice. They appeared similar to the submucosal mesenchymal tumors (SMTs) of the mouse urinary bladder that occurred in a small proportion (1.2%) of mice in various oncogenicity studies, mostly in Swiss Webster and CD-1 mice but at an even lower incidence in other strains (14). At later times, as carcinomas developed, these lesions were no longer seen. At no time were they observed in wt mice.

During the whole process, we observed no difference in weight between *Ptc*^{+/-} and wt mice except by weeks 16–20 when all of the *Ptc*^{+/-} mice had developed carcinomas and were losing weight. Macroscopic examination of other organs at necropsy showed no secondary cancer lesions on any organs, but some anomalies such as mild splenomegaly and rare hepatomegaly were noted in a few animals, wt as well as *Ptc*^{+/-}, mostly after 18 weeks of BBN treatment (15 of 128 wt and 12 of 92 *Ptc*^{+/-}). In eight mice (three wt and five *Ptc*^{+/-}), hydronephrosis due to the presence of large bladder tumors was observed. No anomalies were observed in control mice. To further evaluate the carcinogen exposure and the general toxicity of BBN on mice, we carried out a second carcinogenesis experiment in the same conditions as the first one, on five *Ptc*^{+/-} and six wt mice exposed to BBN during 8 weeks. During that period, no significant difference was observed in water intake and urinary excretion between the two groups of experimental mice, suggesting that carcinogen exposure was identical for the two groups (data not shown). No difference in water intake and urine excretion was observed between experimental mice and a group of six untreated control wt mice. Finally, no significant difference was observed in food intake or weight be-

Table 1 Histopathological characteristics of mouse bladders after BBN^a treatment for different time periods

Genotype	Time (wk)	n	Pre-neopl. ^b	Total TCC	Low-grade TCC		High-grade TCC		Invasive TCC ^e
					Squam. ^c	Squam. ^c	Sarc. ^d		
wt	4	6	0	0	0	0	0	0	0
	8	7	3	0	0	0	0	0	0
	12	9	6	2	1	1	0	0	1
	14	9	8	3	0	0	1	2	3
	16	10	8	5	2	0	0	2	3
	18	10	9	7	0	0	1	6	6
	20	9	7	8	1	1	0	6	7
	24	8	7	8	0	0	1	6	7
<i>Ptc</i> ^{+/-}	4	5	0	0	0	0	0	0	0
	8	7	7	1	0	0	1	0	1
	12	5	4	2	2	0	0	0	1
	14	5	5	4	1	0	1	2	4
	16	11	10	11	4	0	0	7	11
	18	7	6	7	5	0	0	2	4
	20	5	4	5	0	0	0	5	5
	24	3	0	3	0	0	0	3	3

^a BBN, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; Pre-neopl., preneoplastic changes; TCC, transitional cell carcinoma; wt, wild-type; *Ptc*, *Patched* gene.

^b Hyperplasia and atypia.

^c Squam., TCC with squamous differentiation in >50% of the tumor.

^d Sarc., TCC with sarcomatoid differentiation in >50% of the tumor.

^e Invasive TCC was defined as tumor growth in the lamina propria, the muscle layer, the serosa, or neighboring tissues.

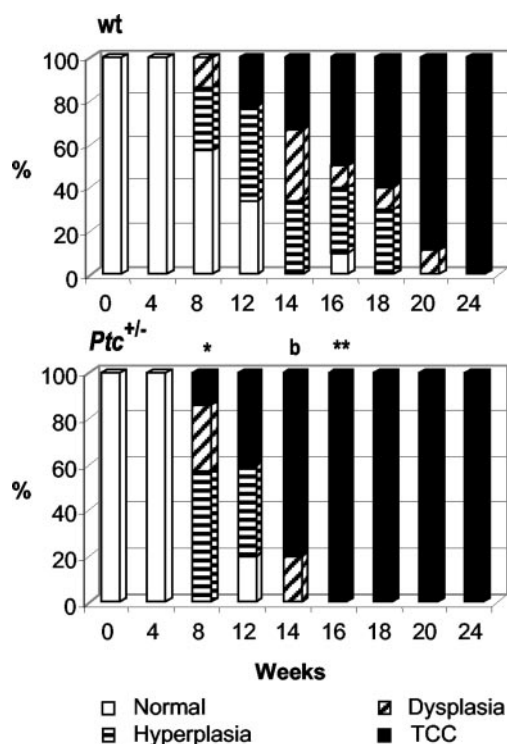


Fig. 2. Histograms showing the proportions of preneoplastic and neoplastic changes in wild-type (wt) and *Ptc*^{+/-} mice after different times of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine treatment. *, $P < 0.05$; **, $P < 0.01$; b, $P < 0.1$ (Jonckheere-Terpstra test).

tween the three groups of mice, suggesting no greater toxic effect of BBN on mutant compared with wt mice (data not shown).

Proliferation and Apoptosis Assays. To determine whether a difference in urothelial cell proliferation between wt and *Ptc*^{+/-} mice could be related to the difference observed in the kinetics of tumorigenesis, we evaluated the proportion of mitotic cells on bladder sections, by staining for phosphohistone H3, which is expressed in cell nuclei, from late G₂ to anaphase (Table 2; Ref. 15). Positive nuclei were strongly stained, and their distribution was heterogeneous and often patchy. Histologically similar regions were counted in *Ptc*^{+/-} and wt mice. After 4 weeks of BBN treatment, a significant increase in proliferation was observed in the normal looking urothelium of the treated mice relative to the controls, both for wt ($P = 0.02$) and *Ptc*^{+/-} mice ($P = 0.03$). Similarly, increased phosphohistone H3 staining was observed in hyperplastic regions ($P = 0.006$ and 0.07 in wt and *Ptc*^{+/-}, respectively) and TCC ($P = 0.005$ and 0.03 in wt and *Ptc*^{+/-}, respectively) compared with normal urothelium. No difference between wt and *Ptc*^{+/-} mice in phosphohistone H3 staining was found after up to 12 weeks of BBN treatment ($0.14 \leq P \leq 0.96$).

The TUNEL reaction was carried out on the same tumors as staining for phosphohistone H3. Between 0 and 3% of intensely stained nuclei were observed, mostly in the upper layer of normal and hyperplastic urothelia and in TCCs, as was reported by Takaba *et al.* (16) in BBN-treated rat bladders. However, we found no difference in the proportion of apoptotic cells between wt and *Ptc*^{+/-} mice at any time ($0.17 < P < 0.89$; data not shown).

Table 2 Percentage of mitotic cells in bladders from wild-type (wt) and *Ptc*^{+/-} mice treated with BBN,^a assayed by staining for phosphohistone H3

Time (wk)	Genotype	Normal		Hyperplasia		TCC	
		n	% cell ^b	n	% cell ^b	n	% cell ^b
Control	wt	4	0.6 ± 0.6	0		0	
Control	<i>Ptc</i> ^{+/-}	4	0.7 ± 0.6	0		0	
4	wt	6	5.5 ± 3.4	0		0	
4	<i>Ptc</i> ^{+/-}	5	5.8 ± 3.5	0		0	
8	wt	7	8.8 ± 4.1	1	13.1	0	
8	<i>Ptc</i> ^{+/-}	5	5.6 ± 2.1	5	12.3 ± 4	0	
12	wt	5	3.5 ± 1.4	4	7.1 ± 1.4	1	12.3
12	<i>Ptc</i> ^{+/-}	4	2.7 ± 0.4	3	8.1 ± 4.9	2	12.8 ± 7.2

^a BBN, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine.

^b % of phosphohistone H3 positive cells ± SD.

Discussion

Various studies have suggested the presence of one or several bladder cancer TSGs on the human chromosome 9q arm (1). The 9q22 locus is a reported candidate TSG locus, making the *PTC* gene at 9q22.3 a possible TSG. Although the role of *PTC* as a TSG has been firmly established in cancers such as BCCs and medulloblastomas, its role in other cancers, such as transitional cell carcinomas, has been suggested but not clearly demonstrated. It has been suggested that characteristics of BCC, such as extensive local growth and weak propensity to metastasize, could be related to disruption of the PTC/sonic hedgehog (SHH) pathway (17). Such characteristics are also typical of superficial TCCs. Moreover, in both tumor types, exogenous carcinogens are likely involved. These observations, as well as the results of our microsatellite mapping of the 9q22 locus and *PTC* expression analysis (3), support the implication of *PTC* anomalies in bladder carcinogenesis.

This study showed a significantly accelerated induction of bladder carcinomas by BBN in *Ptc* heterozygous mutant mice compared with their wt littermates. Exposure to the carcinogen was the same in both groups of mice because no difference was observed in the level of water consumption or in urine excretion between the two groups. No marked toxic effect or cancer lesions were noted on other organs. The *Ptc*^{+/-} mice are known to spontaneously develop medulloblastomas. We observed such tumors but only in mice younger than 6 weeks, before they were under protocol.

The PTC protein is recognized as the membrane receptor of a diffusible protein, SHH (18). According to the generally accepted view of the PTC/SHH pathway, binding of SHH to PTC relieves the repressing effect of PTC on another membrane protein, smoothed (SMO), which through various cytoplasmic components activates a family of transcription factors, the GLI-1 proteins (19). Although much remains to be understood about the complexities and function of the PTC/SHH pathway, several lines of evidence indicate that its activation leads to increased cell proliferation.

Ozaki *et al.* (20) studied BBN-induced bladder carcinogenesis in *p53* heterozygous mutant mice and observed, as in the present study, a higher susceptibility of the mutant mice to develop carcinogen-induced bladder cancer than the wt mice. In their study, they observed a higher level of cell proliferation in the bladders of *p53*^{+/-} mice than in wt mice when exposed to 0.0075% BBN for 4 weeks, as evaluated by 5-bromo-2'-deoxyuridine incorporation. They concluded there was a relationship between increased susceptibility to carcinogenesis and higher cell proliferation, namely that carcinogen-induced DNA damage in key genes initiated carcinogenesis in both wt and *p53*^{+/-} mice equally, but that the *p53* deficit in the latter conferred a growth advantage for the transformed cells. Our own data do not allow such an interpretation. After 4 weeks of treatment with 0.025% BBN, no difference in proliferation was found between wt and *Ptc*^{+/-} mice, although we observed a significantly higher level of urothelial cell proliferation than in untreated controls, as measured by the percentage of nuclei positive for phosphohistone H3. At later times of treatment, we observed a further increase in proliferation in hyperplastic lesions and carcinomas, although, again, no difference was noted between wt and *Ptc*^{+/-} mice. Because these lesions appeared earlier in *Ptc*^{+/-} than in wt mice, total urothelial cell proliferation increased more rapidly in mutant mice. However, it is not possible to conclude that tumors developing in *Ptc*^{+/-} mice have a growth advantage over those induced in wt mice, because proliferation levels remained similar in wt and mutant mice when identical histological features were compared. We may, thus, conclude that

the deficit of one copy of the *Ptc* gene might lead to an increased rate of initiation of malignant transformation.

A decrease in apoptosis in mutant mice could accelerate tumor growth in these. To test that hypothesis, we performed the TUNEL reaction on bladder sections from mice treated with BBN for up to 12 weeks and on control mice. However, we observed no difference in the proportion of apoptotic nuclei between wt and *Ptc*^{+/-} mice. The inhibition of apoptosis thus seems an unlikely mechanism to explain the accelerated growth of tumors in *Ptc*^{+/-} mice.

Apart from the faster appearance of carcinomas, the only clear difference observed between wt and *Ptc*^{+/-} mice under BBN occurred very early in the carcinogenesis process with the presence of foci of stromal cell proliferation in the bladders of all of the *Ptc*^{+/-} mice before the development of carcinomas. These appeared similar to the previously described submucosal mesenchymal tumors of the mouse urinary bladder. These foci of stromal cell proliferation disappeared as carcinomas appeared, but it is impossible for us now to tell whether they are related or not (14).

The downstream effectors of the PTC/SHH pathway, the GLI proteins, are pleiotropic transcription factors. We hypothesize that a deficit of one copy of the *Ptc* gene in the *Ptc*^{+/-} heterozygous mice might be responsible for altered expression of proteins facilitating the local growth and invasion of tumors. This situation is different from that described in BCC in which *PTC* appears to act as a classical TSG for which both copies are inactivated, through deletion and mutation. However, it is similar to human bladder tumors with loss of heterozygosity of the *PTC* marker, in which we observed a decreased expression of PTC (3).

In summary, our data support the hypothesis of the *PTC* gene acting as a TSG in bladder cancer. We observed that mice having only one active copy of the *Ptc* gene develop bladder cancer significantly faster than their wt littermates when submitted to chemical carcinogenesis with BBN. This difference does not appear to be related to a differential exposure to the carcinogen, although a difference in the metabolism of the drug cannot be excluded, because the toxicokinetics and metabolism of BBN in the two types of mice were not evaluated. It does not appear to be related to either increased urothelial proliferation or decreased apoptosis. Further investigation will be needed to elucidate the way in which the PTC/SHH pathway is involved in bladder cancer.

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References

- Knowles MA. The genetics of transitional cell carcinoma: progress and potential clinical application. *BJU Int* 1999;84:412-27.
- Simoneau M, Aboukassim TO, Larue H, Rousseau F, Fradet Y. Four tumor suppressor loci on chromosome 9q in bladder cancer: evidence for two novel candidate regions at 9q22.3 and 9q31. *Oncogene* 1999;18:157-63.
- Aboukassim TO, Larue H, Lemieux P, Rousseau F, Fradet Y. Alteration of the *Patched* locus in superficial bladder cancer. *Oncogene* 2003;22:2967-71.
- Simoneau M, Larue H, Aboukassim TO, Meyer F, Moore L, Fradet Y. Chromosome 9 deletions and recurrence of superficial bladder cancer: identification of four regions of prognostic interest. *Oncogene* 2000;19:6317-23.
- Bale AE, Yu KP. The hedgehog pathway and basal cell carcinomas. *Hum Mol Genet* 2001;10:757-62.
- Zurawel RH, Allen C, Chiappa S, et al. Analysis of PTCH/SMO/SHH pathway genes in medulloblastoma. *Genes Chromosomes Cancer* 2000;27:44-51.
- Goodrich LV, Milenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science (Wash. DC)* 1997;277:1109-13.
- Aszterbaum M, Epstein J, Oro A, et al. Ultraviolet and ionizing radiation enhance the growth of BCCs and trichoblastomas in patched heterozygous knockout mice. *Nat Med* 1999;5:1285-91.

9. Ohtani M, Kakizoe T, Nishio Y, et al. Sequential changes of mouse bladder epithelium during induction of invasive carcinomas by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Cancer Res* 1986;46:2001–4.
10. Hard GC, Durchfeld-Meyer B, Short B, et al. Urinary system. In: Mohr U, editor. International classification of rodent tumors: the mouse. New York: Springer-Verlag; 2001. p. 139–62.
11. Dunn SE, Kari FW, French J, et al. Dietary restriction reduces insulin-like growth factor I levels, which modulates apoptosis, cell proliferation, and tumor progression in p53-deficient mice. *Cancer Res* 1997;57:4667–72.
12. Aubin J, Dery U, Lemieux M, Chailier P, Jeannotte L. Stomach regional specification requires *Hoxa5*-driven mesenchymal-epithelial signaling. *Development (Camb)* 2002;129:4075–87.
13. Gavrieli Y, Sherman Y, Ben Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;119:493–501.
14. Halliwell WH. Submucosal mesenchymal tumors of the mouse urinary bladder. *Toxicol Pathol* 1998;26:128–36.
15. Hendzel MJ, Wei Y, Mancini MA, et al. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G₂ and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma (Berl)* 1997;106:348–60.
16. Takaba K, Saeki K, Suzuki K, Wanibuchi H, Fukushima S. Significant overexpression of metallothionein and cyclin D1 and apoptosis in the early process of rat urinary bladder carcinogenesis induced by treatment with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine or sodium L-ascorbate. *Carcinogenesis (Lond)* 2000;21:691–700.
17. Saldanha G. The Hedgehog signalling pathway and cancer. *J Pathol* 2001;193:427–32.
18. Marigo V, Davey RA, Zuo Y, Cunningham JM, Tabin CJ. Biochemical evidence that patched is the Hedgehog receptor [see comments]. *Nature (Lond)* 1996;384:176–9.
19. Wicking C, Smyth I, Bale A. The hedgehog signalling pathway in tumorigenesis and development. *Oncogene* 1999;18:7844–51.
20. Ozaki K, Sukata T, Yamamoto S, et al. High susceptibility of p53(+/-) knockout mice in *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine urinary bladder carcinogenesis and lack of frequent mutation in residual allele. *Cancer Res* 1998;58:3806–11.