Expression of the Retinoblastoma Gene Product in Bladder Carcinoma Cells Associates with a Low Frequency of Tumor Formation

David W. Goodrich, Yumay Chen, Peter Scully, and Wen-Hwa Lee

Institute of Biotechnology, Center for Molecular Medicine, University of Texas Health Science Center, San Antonio, Texas 78245 [D. W. G., Y. C., W.-H. L.], and University of Nevada School of Medicine, Associated Pathologist Laboratories, Las Vegas, Nevada 89119 [P. S.]

ABSTRACT

Upon inactivation of both alleles of the retinoblastoma gene (RB), individuals develop the intraocular eye tumor, retinoblastoma. The gene encodes a 110,000 phosphorylated nuclear protein that may be involved in regulation of the cell cycle. Besides retinoblastoma, mutations of the gene have been detected in several other types of tumors, including bladder carcinoma. Up to one-third of bladder carcinomas may contain mutations of the RB gene. Introducing the retinoblastoma gene into single retinoblastoma, osteosarcoma, or prostate carcinoma cell lines suppresses their tumorigenicity as assayed in nude mice. We have sought to extend these results by introducing the retinoblastoma gene into multiple bladder carcinoma lines, and analyzing several of the resulting, cloned lines. We have found that inhibition of tumorigenicity, as assayed by tumor growth in nude mice or growth of cells in soft agar, is the only consistent phenotype observed upon re-expression of RB in all bladder carcinoma cells examined. The effect of RB expression on growth and cellular morphology varied depending on the particular parental cell line. We conclude that RB expression generally correlates with reduced tumorigenicity, but not reduced growth rate, in bladder carcinoma cells.

INTRODUCTION

The RB protein is responsible for an inherited predisposition to tumors of the retina (1-3). The p110RB is a phosphorylated nuclear protein that is expressed constitutively in most cultured cells and normal tissues (4-9). Expression of wild-type RB protein, however, is lacking in all retinoblastomas examined to date (7, 10). Since the complete loss of p110RB in susceptible cells presumably leads to tumor growth, RB has been classified as a tumor suppressor gene (11).

Mutations that ablate normal RB expression have also been found in carcinomas of the breast, prostate, bladder, and lung as well as in osteosarcoma, soft-tissue sarcomas, and leukemia (10, 12-25). Unlike retinoblastoma, however, only a fraction of these cancers involve the RB gene, and this fraction varies with the tumor type. About 60% of small cell lung carcinoma cell lines lack expression of RB mRNA, whereas 40 to 50% of osteosarcoma specimens contain mutations of RB (10, 15-17, 21, 24-27). Only a small fraction of tumors or tumor cell lines from breast or prostate carcinoma, and leukemia contain mutations within RB.

Bladder carcinoma has been associated with carriers of RB mutations (28). This observation has led to characterization of the RB gene product in many primary bladder carcinomas and bladder carcinoma cell lines. Alteration of p110RB expression has been observed in from 25% (29) to 33% (10) of bladder carcinoma cell lines. A smaller percentage of invasive primary tumors also lack normal RB protein expression (29, 30). The nature of the mutations causing altered p110RB expression have been determined in some cases. Bladder carcinoma cell line J82 expresses an aberrant protein from a gene containing a point mutation within a splice acceptor (18). The mutation results in the loss of a single exon, comprising 35 amino acids, from the resulting mRNA and protein. In many cases, point mutations rather than gross gene rearrangements are involved in disrupting normal RB expression (10, 18, 29, 31).

Exogenous expression of p110RB in cultured retinoblastoma cells by retrovirus-mediated gene transfer consistently results in the loss of their tumorigenic potential in nude mice (32-34). In addition, re-introduction of the RB gene in an osteosarcoma cell line or a prostate cell line lessens the cells’ ability to form tumors in nude mice (12, 32). RB expression in the osteosarcoma cell line, but not the prostate cell line, initially results in altered cellular morphology and reduction in growth rate. Recently, RB has been expressed by transfection in a single bladder carcinoma cell line (35). Cells containing p110RB have reduced tumorigenicity in nude mice and reduced growth rate in low serum culture. In all these studies, however, only a single representative cell line of each tumor type has been examined.

Since loss of p110RB may be important in a relatively large number of bladder cancers, we wanted to determine whether suppression of tumorigenicity in bladder carcinoma upon expression of RB is a general phenomenon. We have expressed p110RB by retrovirus-mediated gene transfer in 3 different bladder cell lines that originally lacked its expression. By observing the response to exogenous expression of RB in each different, parental cell line, we have determined whether changes in growth rate or morphology are dependent on the particular cell line or are general for bladder carcinoma cells as a whole.

MATERIALS AND METHODS

Cells and Culture Conditions. Bladder carcinoma cell lines HT1376, HT1197, SCABBER, TCC-SUP, and 5637 were obtained from American Type Culture Collection. They were cultured in Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. J82, also obtained from American Type Culture Collection, was maintained in Eagle's minimum essential medium supplemented with nonessential amino acids and 10% fetal calf serum. Sera were supplied by JRH Biosciences (Lenexa, KS), whereas other culture components were purchased from Cell Culture Laboratories (Cleveland, OH). Media were supplemented with 800 µg/ml G418 (Sigma, St. Louis, MO) for selection of neomycin resistance.

Growth rates were analyzed after expansion and screening, approximately 2-4 months after the initial infection. About 1 × 10^6 to 5 × 10^6 cells were seeded per 60-mm culture dish in normal growth media. Every 2 days thereafter, cells from each of 3 dishes for each cell line were counted in duplicate with a hemocytometer after trypsinization. The 6 counts were averaged and the log of this value was plotted versus the time of incubation.

Isolation of RB Expressing Cell Lines. Construction of amphotropic retroviral vectors carrying the RB complementary DNA and the isola-
tion of infectious viral stocks have been described elsewhere (12, 32). Approximately $5 \times 10^5$ bladder cells were incubated with $5 \times 10^5$ to $1 \times 10^6$ infectious units over a 24-h period. Three days after infection, G418 was added to the media for 2–4 weeks in order to select cells containing the virus.

Screening of HT1376- and 5637-derived G418-resistant cell lines for the expression of $p110^{RB}$ was performed by immunoblotting and detection using anti-RB mouse monoclonal antibody 245 and rabbit polyclonal antibodies.47 or R2 essentially as described (36). J82-derived cell lines were screened by both immunoblotting cell lysates with anti-RB rabbit polyclonal antibodies R1 or R2 as above, and by mixing aliquots of the cell lysates with purified T antigen and immunoprecipitating with anti-T antibody 419. After SDS-PAGE and electrotransfer, protein blots were probed with both anti-RB antibody 245 and anti-T antigen antibody 419. Cell lysates were prepared by extraction with a buffer containing 50 mM Tris-Cl, pH 7.4, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 50 μg/ml aprotinin, 50 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride.

Analysis of Tumorigenicity. Tumorigenicity assays were performed in nude mice as described (12, 32, 37). From $1 \times 10^5$ to $5 \times 10^5$ cells from HT1376-derived lines, or $5 \times 10^6$ to $1 \times 10^7$ cells from 5637-derived lines were injected s.c. into the flanks of athymic, nude mice. Tumor formation and tumor size were monitored weekly for 2 to 3 months after infection. Mice were sacrificed and the tumors removed for measurement.

Tumor samples used for protein analysis were quick frozen in liquid nitrogen, ground into a fine powder while frozen, then extracted as described above. After clarification of the lysate by centrifugation, anti-RB polyclonal antibody 47 was used to immunoprecipitate the protein. Aliquots from each lysate were also immunoprecipitated with a rabbit polyclonal antibody raised against a DCC gene fusion protein.4 Since this antibody reacts with a $M_r$, 150,000-160,000 protein that is typically less abundant than $p110^{RB}$, we used it to control for possible degradation of the protein preparation during extraction and immunoprecipitation. After SDS-PAGE and transfer, the protein blots were probed with anti-RB antibody 245 or the polyclonal anti-DCC antibody.

Soft agar colony formation assays were done essentially as described (32). Equal numbers of cells ($1 \times 10^5$) from each of the indicated cell lines were plated in duplicate in 0.3% agar. After 30 days of incubation at 37°C, colonies containing at least 50 cells were counted, and the values for each of the duplicate plates averaged.

RESULTS

Introduction of the RB Gene by Retrovirus Mediated Gene Transfer. Several bladder carcinoma cell lines were screened for expression of $p110^{RB}$ by immune precipitation and Western blotting. Cell lines TCC-SUP, 5637, and HT1376 lacked detectable protein of the size expected for the RB gene product (Fig. 1). Only TCC-SUP had gross changes detectable by Southern blot in RB (10). Since $p110^{RB}$ could be detected in any of these cell lines, we considered them to contain null alleles of RB. Cell line J82 was previously shown to contain an RB gene with a point mutation that gave rise to a slightly smaller, unphosphorylated protein lacking exon 21 (18). Although J82 contained RB protein, it was probably nonfunctional since it did not bind SV40 T antigen, it could not be phosphorylated, and it was not tethered to the nucleus (18, 38). Bladder carcinoma cell lines HT1197 and SCABER contained $p110^{RB}$. Cell lines 5637, HT1376, and J82 were used as recipients for expression of exogenous RB by retrovirus mediated gene transfer.

The bladder carcinoma cells were infected by a retroviral stock that had been collected from an amphotropic retroviral packaging cell line containing the RB gene within a retroviral replication unit (12, 32, 34). Since the retroviral genome also included the gene for neomycin acetyltransferase, bulk infected cells were selected with G418 and resistant cells were cloned directly or by limiting dilution. The cell clones were expanded and then screened for expression of the $M_r$, 110,000 RB protein.

Since parental cell lines 5637 and HT1376 did not express detectable $p110^{RB}$, virus infected cell lines were screened by immune precipitation and Western blotting with antibodies directed against the RB protein. At least 25 cell lines were cloned from each of the 5637 and HT1376 infections. Of 10 5637-derived cell lines tested, $p110^{RB}$ could be detected in 8 (Fig. 2A). Highly phosphorylated forms of the protein were also observed. Expression of $p110^{RB}$ was detected in 7 of the 9 HT1376 cell lines analyzed (Fig. 2B).

Horowitz et al. (18) have reported that cell line J82 has an endogenous, mutant RB protein that does not bind SV40 T antigen and that is slightly smaller in size than $p110^{RB}$. Therefore, we screened J82-derived cell lines for expression of $p110^{RB}$ by immunoprecipitating lysates with anti-RB antibodies as above, and by immunoprecipitating cell lysates with anti-T antigen antibody after addition of purified T antigen. The presence of $p110^{RB}$ was indicated by the presence of slower migrating, phosphorylated species of RB protein and by co-immunoprecipitation with T antigen. Immune precipitates were resolved by SDS-PAGE, immunoblotted, and RB and T antigen proteins detected by probing the blot with the appropriate antibodies. Fig. 2C shows the analysis of 4 representative cell lines; 2 of these cell lines (Fig. 2C, Lanes 5 and 9) contain highly phosphorylated forms of $p110^{RB}$. These 2 cell lines also contained an unphosphorylated form of RB that was slightly larger than the endogenous, mutant protein, and was capable of being co-precipitated by T antigen antibody (Fig. 2C, Lanes 6 and 10). The remaining lines (Fig. 2C, Lanes 3, 4, 7, 8, 11, and 12), do not contain phosphorylated $p110^{RB}$ and do not co-immunoprecipitate with SV40 T antigen. Of 36 J82, neomycin resistant cell lines cloned, only 2 stably expressed $p110^{RB}$ (data not shown).

Morality and Growth Properties of Bladder Carcinoma Cells Reconstituted for Expression of $p110^{RB}$. Re-introduction of the RB gene has significant effects on the cellular morphology of an osteosarcoma cell line, but almost no difference in morphology was observed upon expression of $p110^{RB}$ in a prostate or bladder carcinoma cell line (12, 32, 35). Bladder carcinoma cell lines expressing $p110^{RB}$ isolated in this study showed little morphological change when compared to the 3 parental bladder carcinoma lines (Fig. 3). Initially, a small increase in cell size and a lower saturation density was observed.
A.

B.

C.

Fig. 2. Screening of cloned bladder carcinoma cell lines for re-expression of exogenous, p110RB. Cell lines obtained after infection with the RB gene-containing retrovirus were expanded and protein from such lines screened for the presence of p110RB as described in "Materials and Methods." A, cell lines derived from bladder carcinoma line 5637. The following cell lines are shown: Lane 1, 5637-RB-27; Lane 2, 5637-RB-23; Lane 3, 5637-RB-22; Lane 4, 5637-RB-15; Lane 5, 5637-RB-14; Lane 6, 5637-RB-12; Lane 7, 5637-RB-11; Lane 8, 5637-RB-9; Lane 9, 5637-RB-6; Lane 10, 5637-RB-5; Lane 11, parental 5637. B, cell lines from the HT1376 bladder carcinoma line: Lane 1, HT1376 parental; Lane 2, HT1376-RB-B; Lane 3, HT1376-RB-D; Lane 4, HT1376-RB-G; Lane 5, HT1376-RB-H; Lane 6, HT1376-RB-J; Lane 7, HT1376-RB-L; Lane 8, HT1376-RB-V; Lane 9, HT1376-RB-W; Lane 10, HT1376-RB-X. C, results of analysis of 4 cell lines made from the J82 cell line. In this case protein was immune precipitated with either anti-RB antibody (Lanes 1, 3, 5, 7, 9, and 11) or anti-T antigen antibody (Lanes 2, 4, 6, 8, 10, and 12). Purified T antigen was added to lysates in Lanes 4, 6, 8, 10, and 12. Immune precipitates were resolved by SDS-PAGE, Western blotted, and probed with both anti-RB and anti-T antigen antibodies. The following cells were examined: Lanes 1 and 2 are from COS1 and serve as a positive control; Lanes 3 and 4 are from line J82-1B3; Lanes 5 and 6 are from line J82-1A5; Lanes 7 and 8 are from line J82-2A3; Lanes 9 and 10 are from line J82-2C2; Lanes 11 and 12 are from J82 cells infected with a retrovirus lacking the RB gene. In all cases the positions of RB and/or T antigen proteins are indicated.

Fig. 3. Effect of expression of p110RB on the morphology of bladder carcinoma cells. Cell lines expressing p110RB were examined by phase contrast microscopy and compared to cell lines that failed to make RB protein. a, b, and c, morphology of 5637, HT1376, and J82-derived cell lines, respectively, that did not contain p110RB. d, e, and f, p110RB expressing cell lines from 5637, HT1376, and J82, respectively. All cells were photographed with a x 40 objective, however, c and f were further magnified photographically.
cell lines. Cells were plated at 5-6 x 10⁶ cells per 60-mm culture dish and 3 dishes were counted approximately every 48 h thereafter. The average log cell number was indistinguishable from those lacking RB protein. The results from cell lines made from HTI376. D. parental line HTI376; O, cell from the 5637-derived cell lines. G D, parental line 5637-2; •¿ •¿. line 5637 upon p110RB expression, however this phenotypic change was not stable and after several passages p110RB-containing cells were indistinguishable from those lacking RB protein. The morphological heterogeneity observed between individual cell lines was greater than the average difference between p110RB positive and p110RB negative cell lines.

The growth rates of osteosarcoma cells expressing p110RB were significantly lower than cells that lacked RB, while the growth rates of prostate carcinoma cells were similar whether they expressed p110RB or not (12, 32). Takahashi et al. (35) reported that the growth rates of 2 RB-expressing bladder carcinoma cell lines made by transfection were slower than parental lines when grown in 3% fetal calf serum. The growth rates of representative bladder carcinoma cell lines made here were also determined. No consistent growth rate difference was observed in either 5637- or HTI376-derived cell lines between those containing p110RB and those that lack p110RB when grown in 10% fetal calf serum (Fig. 4, A and B). Although the growth rates of these individual cell lines varied considerably in 0.5% fetal calf serum, there was no correlation with expression of p110RB (data not shown). J82 cells that stably expressed wild-type RB protein did have a consistently slower growth rate in 10% fetal calf serum than either parental J82 cells or virus-infected cell lines that lacked p110RB (Fig. 4C). The decrease in growth rate upon expression of p110RB in J82 cells is one possible explanation as to why a very low percentage of these cloned cell lines stably expressed RB protein.

Tumorigenic Potential of Bladder Carcinoma Cells Reconstituted for Expression of p110RB. To test the effect of p110RB expression on the tumorigenic potential of bladder carcinoma cells, we s.c. injected cells from representative cell lines into the flanks of athymic nude mice. HTI376 was the most tumorigenic of the 3 parental cell lines; 5 x 10⁶ cells, the lowest dose of injected cells tested, were capable of forming a detectable tumor within 2 months. Approximately 1 x 10⁶ 5637 injected cells were required for consistent tumor formation within 2 to 3 months. Parental J82 cells, in contrast, did not form tumors even upon injection in high numbers. For subsequent assays, 1 x 10⁶ to 5 x 10⁶ cells from HTI376-derived lines and 5 x 10⁶ to 1 x 10⁷ cells from 5637-derived lines were injected.

Four 5637-derived cloned cell lines that express p110RB were tested for tumorigenicity in nude mice in addition to one virus-infected cell line that lacked p110RB and the parental 5637 line. The tumor frequency and size caused by injecting RB expressing cells was low compared to cells lacking p110RB (Table 1). Seven virus-infected HTI376 cloned cell lines that express p110RB and one that did not, in addition to HTI376, were also injected into nude mice. Excepting one cell line, HTI376-RB-D, the results were similar to those of the 5637-derived bladder cell lines. The frequency and/or volume of tumors that arose were generally lower upon expression of RB protein. Tumorigenicity of the parental cell line HTI376 was greater than the cloned cell line lacking p110RB, HTI376-RB-V. The decreased heterogeneity of the cells in the cloned line and its generally slow growth rate in culture (Fig. 4) may have been responsible for the small size of the tumors formed. Cell line RB-D did form moderately sized tumors frequently upon injection even though it originally expressed apparently wild-type RB protein (see "Discussion").

Tumors arising from bladder carcinoma cell lines expressing p110RB may be due to the outgrowth of a small number of cells that no longer make the protein. To test this, multiple tumors that grew from each of 3 RB-expressing lines, including HTI376-RB-D, were analyzed for p110RB. Protein from tumor samples was extracted, immunoprecipitated, resolved by SDS-PAGE, blotted, and probed for the presence of RB protein. No protein of the size expected for p110RB was detected in tumors that resulted from injection of HTI376, HTI376-RB-B, HTI376-RB-D, or 5637-RB-B (Fig. 5A). To control for possible protein degradation, aliquots of the tumor lysates were processed as above using an anti-DCC antibody that reacts with a M, 160,000 protein⁴ that is typically less abundant than p110RB. This protein was detected in each tumor sample (Fig. 5B).

The J82 cell line and derivative cloned lines were not tumorigenic upon s.c. injection into athymic, nude mice. As an alternative assay for their neoplastic potential, these cells were assayed for their ability to grow in soft agar. Two cloned cell lines that expressed p110RB and one cloned cell line that did not express p110RB, in addition to the J82 parental line, were assayed. A correlation was observed between expression of wild-type RB protein and an inability to grow in soft agar; the number of colonies growing from cells containing p110RB was 7- to 20-fold less than from cells not containing p110RB (Table 2). The differences in the growth rates in soft agar upon expression of wild-type RB exceeded the differences observed in growth rates in culture.

### Table 1 Tumorigenicity in nude mice of bladder carcinoma cells reconstituted for RB expression

<table>
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<th>Cell line</th>
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<th>Tumors*</th>
<th>Volume (mm³)*</th>
<th>RB protein</th>
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<tr>
<td>HTI376</td>
<td>18</td>
<td>17</td>
<td>1000</td>
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<td>-</td>
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<td>250</td>
<td>+</td>
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<td>5</td>
<td>400</td>
<td>+</td>
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<td>4</td>
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</tr>
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<td>1</td>
<td>80</td>
<td>+</td>
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</table>

* Approximately 1 x 10⁶ to 1 x 10⁷ cells (see text) were injected s.c. into the flanks of nude mice.

* Tumors were scored and measured at 2 to 3 months after injection. The sizes of the tumors are the average of all specimens examined.
RB suppresses tumorigenicity of bladder cells

In this study we have explored the effects of re-introduction, by retrovirus-mediated gene transfer, of a wild-type RB gene on bladder carcinoma cells. Previous studies introducing RB into retinoblastoma, osteosarcoma, prostate, and bladder carcinoma cells made p110RB expressing cells from a single cell line of each type (12, 32, 33, 35). Although loss of tumorigenicity upon expression of p110RB was a consistent observation of all these studies, the particular changes in tumorigenicity, morphology, or growth rate reported may have depended, in part, on the particular cell line chosen rather than the cancer cell type. In the one previous study with bladder carcinoma cells, Takahashi et al. (35) introduced p110RB expression vector by transfection that can result in very high levels of protein. Furthermore, only 2 independent cell lines containing p110RB were analyzed for tumorigenicity in nude mice. Here we have analyzed multiple RB-expressing lines derived from each of 3 independent bladder carcinoma cell lines.

In 2 cases, HT1376 and 5637, a high proportion of selected, cloned cell lines stably expressed the wild-type RB protein. In J82, however, a low percentage of selected, cloned lines contained p110RB. The level of expression in each cell line was comparable to that of endogenous, wild-type RB in normal conditions. One possible explanation for the low number of J82 cells that stably express p110RB was that such expression placed these cells at a selective growth disadvantage under the culture conditions used. In fact, the growth rate of J82 cells upon expression of RB was retarded, whereas the presence of p110RB in HT1376 or 5637 cells had no detectable effect on cell proliferation. Our results indicate that the effects of RB expression on retardation of growth rate is dependent on the particular cell line examined; RB is not a general suppressor of proliferation in bladder carcinoma cells. Also, the morphology of cells containing p110RB, derived from each parental cell line, was not significantly different from cells lacking p110RB. Although a slight enlargement in cell size and a lower saturation density of cells containing RB was initially observed, these properties were not stable over passage in culture.

Although the effects on growth rate in culture varied among the 3 bladder cell lines studied here, each of the cell lines was less tumorigenic once they expressed p110RB. That expression of RB inhibited tumorigenicity was demonstrated by the low frequency of tumor formation in nude mice, the relatively small tumor size and lack of detectable p110RB in the tumors that did arise, or the inability to form colonies in soft agar. One cloned bladder carcinoma cell line that contained p110RB, RB-D, did give rise to tumors relatively frequently. However, no RB protein could be detected in the tumors that arose from injection of these cells, suggesting that expression of RB was particularly unstable in this line. Unstable expression of RB may have contributed to the relatively high frequency of tumor formation; such instability would not have been readily detectable in culture since there was no growth disadvantage upon expression of p110RB. The independence of the effect p110RB on growth rate in culture and in nude mice was pointed out particularly by examination of HT1376-derived cell lines. RB-V, although lacking p110RB, had one of the slowest growth rates in culture examined. Several RB positive cell lines had faster growth rates. However, RB-V was significantly more tumorigenic in nude mice, although the tumors grew relatively slowly when compared with the parental cell line. These findings, together with previous results, suggested that RB acts as a general suppressor of tumorigenicity, but not growth rate in culture, in bladder carcinoma cells.

Our results are generally consistent with the previous report of suppression of tumorigenicity in a bladder cell line by expression of RB (35). However, we do not detect any p110RB in tumors that occasionally arose from cell lines that originally expressed RB. This difference could be accounted for by the different parental bladder carcinoma lines used. The degree of suppression of tumorigenicity conferred upon p110RB expression may vary between bladder carcinoma cell lines. Alternatively, the stability of expression after injection into nude mice might be different in these experiments because of the different methods of gene transfer used.

Although bladder carcinoma cells have reduced tumorigenicity after re-introduction of RB, the loss of RB function by mutation is not involved in the genesis of all bladder carcinomas. The RB gene has been linked to control of cell cycle progression and hence proliferation (39). It is possible that loss of RB function may induce unscheduled cell proliferation, and that unscheduled proliferation may increase the rate of fixation of additional mutations important for bladder carcinogenesis. Hence, loss of RB function could promote tumorigenesis, when it does occur, in an indirect way; in any case, pathways to bladder cancer exist that are independent of RB. It would be interesting to know whether there are any clinical differences...
between bladder carcinomas that lack RB function and those that do not.

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REFERENCES


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