Promoter deletion and loss of retinoblastoma gene expression in human prostate carcinoma

(tumor suppression/immunohistochemistry/polymerase chain reaction)

ROBERT BOOKSTEIN*, PASCALE RIO*, STEVEN A. MADREPERLA†, FRANK HONG*, CRAIG ALLRED‡, WILLIAM E. GRIZZLE§, AND WEN-HWA LEE*

*Department of Pathology, Center for Molecular Genetics, and Cancer Center, University of California at San Diego, La Jolla, CA 92093-0612; ‡Department of Pathology, The University of Texas Health Sciences Center at San Antonio, San Antonio, TX 78284; and §Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294

Communicated by Helen M. Ranney, July 16, 1990 (received for review April 6, 1990)

ABSTRACT Mutational inactivation of the retinoblastoma gene (RB) is found in all retinoblastomas and in a subset of other human neoplasms, including sarcomas of bone or soft tissue and carcinomas of lung or breast. Exogenous copies of wild-type RB have been shown to suppress the tumorigenicity of several types of tumor cells with endogenous RB mutations, including a previously described human prostatic carcinoma cell line. To further support a role for RB inactivation in the genesis of prostate cancer, seven primary or metastatic prostatic carcinoma specimens were examined for evidence of RB mutation. By the use of immunoblot analysis and immunostaining of histologic sections, RB-encoded protein was readily detected in tumor cells of five specimens, was equivocally detected in one specimen, and was apparently absent from tumor cells of one specimen. RB mutations in the latter case were precisely characterized as (i) a deletion of 103 nucleotides containing transcriptional start sites and (ii) loss of the second RB allele. The 103-base-pair deletion was sufficient to abolish the promoter activity of upstream DNA sequences in a heterologous expression system. These results (i) demonstrate that RB can be inactivated in vivo by mutation of its promoter, (ii) confirm the existence of RB mutations in some human prostatic carcinomas, and (iii) suggest the use of immunohistochemical methods to screen for RB mutations in clinical samples of common adult neoplasms.

Prostate carcinoma is the most common cancer in men (1). Despite its high incidence, the genetic alterations affecting prostatic cancer cells are poorly understood. Genetic analyses of common adult neoplasms, as well as of several unusual childhood tumors, have suggested that the cancer phenotype is broadly determined by two classes of genes, oncogenes and tumor suppressor genes, that are functionally opposed (2). ras and myc oncogenes, activated by mutation or overexpression, have an uncertain role in the genesis of prostatic neoplasms (3–5), although a mouse model for multistage carcinogenesis was created by introducing these oncogenes into prostatic precursor cells (6). Deletion of chromosome region 10q24 has been identified in several prostate carcinomas; karyotypes of these tumors are otherwise intratably complex (7). Loss of heterozygosity of specific genetic markers, suggestive of the inactivation of nearby tumor suppressor genes, has not been reported in prostate cancer.

Of the few tumor suppressor genes cloned to date, the retinoblastoma gene (RB) is certainly prototypic. Both alleles of this gene are invariably mutated in retinoblastoma, a tumor of childhood that arises in the immature retina (8). In hereditary retinoblastoma, one mutation is inherited in the germ line, whereas nonhereditary tumors require two somatic mutations, as predicted by Knudson (9). RB mutations are also found in a subset of osteosarcomas, soft-tissue sarcomas, and carcinomas of breast and lung, suggesting a broad role for RB inactivation in the genesis of human tumors (8). Finally, restoration of the normal RB gene product, pp110RB, into pp110RB-deficient (RB−) tumor cells alters several aspects of their neoplastic phenotype, including suppression of tumorigenicity in nude mice (10, 11). Because of these properties, RB is a model for studying other candidate tumor suppressor genes.

In a previous study of three human prostate carcinoma cell lines, one was found to express a mutated RB mRNA and an abnormally small RB protein that was functionally inactive in tumor suppression (11). To establish the existence of RB mutations in native prostate carcinomas, primary or metastatic tumor specimens were screened for loss of RB protein expression. Two tumors were found to have severely reduced or undetectable amounts of RB protein by immunoblot analysis and by immunostaining of histologic sections. Direct detection of mutated RB alleles is hindered by the large size (=200 kilobases) and structural complexity (27 exons) of this gene (12). Nevertheless, a detailed genetic analysis of one tumor revealed both mutations leading to RB inactivation: (i) a 103-base-pair (bp) deletion that abolished activity of the RB promoter and (ii) loss of the second normal RB allele.

MATERIALS AND METHODS

Origin of Prostate Carcinoma Specimens. Unfixed tumor samples and benign prostate tissue were collected in conjunction with surgical procedures or autopsies, and were stored at −70°C until used. Tumor 7 was obtained from a patient (patient 4) whose neoplasm was included in a previous study of neuroendocrine differentiation in prostatic carcinomas (13).

Immunoblot Analysis. Frozen samples were pulverized in mortars on dry ice and lysed in 1 ml of ice-cold lysis buffer (50 mM Hepes, pH 7.5/150 mM NaCl/0.1% Nonidet P-40/50 mM NaF/1 mM phenylmethylsulfonyl fluoride). After clarification (20,000 × g for 5 min), lysates were equilibrated for total protein content (MicroBCA kit, Pierce) and supplemented with protease inhibitors aprotinin (Boehringer Mannheim) and leupeptin (Sigma) at 10 μM and 50 μM, respectively. Lysates were immunoprecipitated with rabbit polyclonal anti-RB IgG (1.5 μg/ml) (14), anti-esterase D IgG (1.0 μg/ml) (15), or anti-c-Ab1 IgG (1.0 μg/ml) (16), followed by protein A-Sepharose CL-4B beads (Pharmacia LKB), as

Abbreviations: VNTR, variable number of tandem repeats; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction.

*Present address: Wilmer Eye Institute, Johns Hopkins Hospital, Baltimore, MD 21205.

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were followed by RB, esterase D, or c-Ab proteins, respectively.

Immunohistochemistry. Immunostaining methods were adapted from Stoner et al. (20) and Mayer and Walker (21). In brief, 10-μm-thick cryosections of frozen tumor fragments were immediately fixed in ice-cold acetone for 10 min and then air-dried. Slides were washed for 10 min in PBS (10 mM sodium phosphate/0.15 M NaCl, pH 7.2), blocked for 30 min in 3% (vol/vol) normal goat serum in PBS (NGS/PBS), and then incubated overnight in NGS/PBS with or without affinity-purified rabbit polyclonal anti-RB.47 IgG (0.5 μg/ml) (22). Washing and blocking steps were repeated, and slides were incubated for 1 hr with biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200 in NGS/PBS. Slides were washed, incubated for 45 min in ABC reagent (Vector Laboratories), washed again, and then developed in 0.02% 3,3'-diaminobenzidine tetrahydrochloride (Sigma)/0.01% H2O2/PBS. Nuclear counterstaining, if required, was done with 0.25% aqueous methyl green (Sigma), followed by dehydration and permanent mounting.

DNA Extraction. Frozen tumor fragments were serially cryosectioned at 10-μm intervals. The first and last sections were extracted by routine histology. Genomic DNA was extracted by the routine histology sections (~0.02 cm² of total tissue) by standard procedures (23).

Genomic Polymerase Chain Reaction (PCR). RB promoter DNA fragments were amplified by mixing primers Rh-Pr1 (500 ng; 5'-TGACAGGAAATGATTCCGC-3') and Rh-4 (500 ng; 5'-TCCGACAGTCCCTACA-3'), 1 μg of genomic DNA, 10 μl of dimethyl sulfoxide, and 1 unit of Thermus aquaticus polymerase (Cetus) with PCR buffer (50 mM KCl/10 mM Tris-HCl, pH 8.3/1.5 mM MgCl2/0.01% gelatin) and 200 μM dATP, 200 μM dCTP, 200 μM dGTP, and 200 μM dTTP (dNTPs) in a total volume of 100 μl. An alternative pair of primers 5'-GTAAGAGCCTC (Rh-Pro-1) and 5'-TAAAGCTTCTC (Rh-4) had Ssr I- or HindIII-digestible linker sequences, respectively, at their 5' ends. Mixtures were processed through 33 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by a single 5-min extension step in a programmable heat block (Ercopim, San Diego, CA). The highly polymorphic region in intron 20 of the RB gene (24) was amplified by mixing primers Rh-35 (250 ng; 5'-GAACGCTTCTGCTAGG-3') and VNTR20 (250 ng; 5'-AATTAACAGGTTGTTGGG-3'), 1 μg of genomic DNA, 10 μl of dimethyl sulfoxide, and 1 unit of Thermus aquaticus polymerase with PCR buffer and dNTPs in 100 μl. An additional 10–50 ng of Rh-35 radiolabeled with [γ-32P]ATP and T4 kinase (25) was included as a tracer. Reaction conditions were 28 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 30 sec, followed by a single 5-min extension step. The resulting allele sizes were approximately 400 base pairs (bp). PCR products (5 μl) were denatured by boiling with equal volumes of 0.37% EDTA sequencing stop buffer and loaded on a 6% polyacrylamide sequencing gel as described (24).

Direct Sequencing. PCR-amplified DNA was purified on 8% polyacrylamide gels and Elutip-d columns (Schleicher & Schuell). Primers Rh-4 (see above) and PR-1 (5'-TCTGGACCTAGCTAG-3') were radiolabeled with [γ-32P]ATP and T4 kinase and purified by spin-column chromatography (25) with Bio-Gel P-30 (Bio-Rad). Sequencing reactions were performed with reagents supplied in a T7 polymerase sequencing kit (Pharmacia LKB). Approximately 200 ng of template DNA was mixed with 12 ng of primer and 1 μl of dimethyl sulfoxide in 15 μl and denatured at 95°C for 3 min. After quickly chilling this mixture on ice, 2 μl of annealing buffer was added, and the mixture was annealed at 37°C for 20 min and then at room temperature for 10 min. Instead of labeling reagents, 1 μl of 4 μM dATP and 2 μl of diluted T7 polymerase (1.5 units/μl) were added. The mixture was immediately distributed into four termination reaction tubes (4.5 μl per tube), and the procedure was continued according to the kit instructions. Reaction products were separated on a 6% polyacrylamide sequencing gel, and the gel was autoradiographed.

**RB Promoter-Driven Chloramphenicol Acetyltransferase (CAT) Expression Assay.** Tumor 7 DNA was amplified with linker-containing primers, and the aberrant promoter fragment ΔSH was digested with HindIII and Ssr I and gel-purified as described above. Plasmid pRB-CAT2, containing the normal RB promoter driving the bacterial CAT gene (12), was linearized with Ssr I and partially digested with HindIII. A 4.4-kilobase fragment (pRB-CAT2 minus the RB promoter) was purified from agarose gels and ligated to ΔSH, resulting in pASpCAT. CV-1 cells were transfected with pASpCAT DNA and other previously characterized control plasmids as described (12); promoter insertions in all plasmids had similar 3' termini but varied at their 5' ends. Assays for transient expression of CAT and luciferase (an internal control for transfection efficiency) were performed as described by Hong et al. (12).

## RESULTS

**RB Protein Expression in Human Prostate Carcinomas.** RB mRNA is expressed constitutively in most nonneoplastic tissues (26, 27) and encodes a nuclear phosphoprotein of apparent M, 110,000–116,000, depending on the extent of phosphorylation (28). Loss of RB protein expression, or expression of shortened RB polypeptides, as determined by immunoblot analysis, has proved to be a sensitive indicator of RB mutation in several types of cultured tumor cells (11, 14, 17, 28–30), and similar methods might be applied to native tumor specimens. To minimize the problem of contaminating nonneoplastic elements, which could obscure the lack of RB protein in tumor cells, metastatic or bulky prostate tumors were favored for initial examination (Table 1). Approximately equal quantities of normal-sized RB protein were detected in benign prostate tissue (data not shown) and in three tumor samples, whereas RB protein content was significantly reduced in one specimen and was undetectable in lysates of two others (Table 1). To confirm the specific loss of RB protein from tumor cells, immunohistochemical methodology was used.

### Table 1. Summary of RB protein expression in human prostate carcinoma specimens

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Source</th>
<th>Primary or metastatic site</th>
<th>pp110RB</th>
<th>% tumor cells staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Biopsy</td>
<td>Primary</td>
<td>ND</td>
<td>&gt;90</td>
</tr>
<tr>
<td>2</td>
<td>Biopsy</td>
<td>Lymph node</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>Autopsy</td>
<td>Liver</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>Autopsy</td>
<td>Pelvis*</td>
<td>++</td>
<td>&gt;90</td>
</tr>
<tr>
<td>5</td>
<td>Autopsy</td>
<td>Liver</td>
<td>+</td>
<td>~30</td>
</tr>
<tr>
<td>6</td>
<td>Autopsy</td>
<td>Bone</td>
<td>-</td>
<td>&lt;5*</td>
</tr>
<tr>
<td>7</td>
<td>Autopsy</td>
<td>Pelvis*</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

pp110RB was quantitated by Western blot analysis. + and - indicate presence or absence of protein, respectively.

**Direct extension to pelvic soft tissues.**

*See text.
ods were developed to detect RB protein in histologic sections (Fig. 1). Distinct nuclear staining was observed in benign prostatic epithelial cells (Fig. 1a) and in tumor cells of several prostate carcinomas (Table 1). Nearly all nuclei were stained in tumors 1 and 4 (Fig. 1 b and c), whereas smaller fractions of nuclei in tumors 5 and 6 were positive. The fraction of immunostained cells, therefore, roughly correlated with the amounts of RB protein detected by immunoblot analysis. Neoplastic cells in tumor 7 were completely unstained; the few positively staining nuclei present were in delicate linear arrays and thus comprised tumor microvasculature (Fig. 1d). In summary, tumor cells of four prostate carcinomas contained apparently normal RB protein by immunoblot analysis and/or immunostaining, one (tumor 7) completely lacked detectable RB protein expression, and two had intermediate results (see Discussion).

**Homozygous Deletion Within the RB Promoter Region in a Prostate Carcinoma.** To confirm that loss of RB protein expression in prostate carcinoma could result from RB mutation, we sought to detect such mutations in at least one RB-protein-deficient tumor specimen described above. Tumor 7 was considered the best candidate. Southern blot analysis of genomic DNA from this tumor suggested that its RB alleles were grossly intact (data not shown). As an alternative strategy, multiple pairs of oligonucleotide primers flanking RB coding regions have been used to amplify tumor DNA in vitro; mutations are subsequently detected by direct sequencing (31). Because the RB promoter region as defined by Hong et al. (12) was a plausible site for inactivating mutations, tumor 7 and control DNA samples were amplified in vitro with two primers flanking the upstream regulatory sequence of RB (Fig. 2A). PCR products with template DNA from prostate carcinoma cell line DU145 (11) and from tumors 3 and 4 yielded amplified fragments of the expected size (422 bp). However, PCR products with template DNA from two portions of tumor 7 contained two distinct bands, one normal-sized and the other about 100 bp smaller (Fig. 2A, lanes 3 and 4). As assessed by histologic sections, one template DNA sample (lane 3) was derived from a region of confluent tumor cells and minimal stroma, whereas the other (lane 4) was derived from mostly nonneoplastic tissue containing just a small nest of tumor cells. Correlatively, the quantity ratios of normal-sized and small amplified fragments were about 1:10 and 10:1 in PCR products with these two template samples, respectively. Detection of an aberrantly

**Fig. 2.** RB mutations in a prostate carcinoma specimen. (A) Deletion within the RB promoter. DNA from prostate carcinoma cell line DU145 (lane 2), from two separate portions of tumor 7 (lanes 3 and 4), and from tumors 3 and 4 (lanes 5 and 6, respectively) was amplified in vitro with two oligonucleotide primers flanking the RB promoter. PCR products were separated on an 8% polyacrylamide gel and visualized by ethidium bromide staining. Lane 1 was a no-template control. Aberrant shortened fragments were apparent only in DNA from tumor 7; exact fragment sizes (right) were inferred from results presented in Fig. 3. Lane M contains the size markers. (B) Loss of heterozygosity of a VNTR polymorphism within RB. DNA samples from tumor 7 (lanes 3 and 4 in A) were amplified in vitro with primers flanking a VNTR region in RB intron 20 (24). The smaller VNTR allele apparent in one DNA sample (left lane) was markedly reduced in the other sample (right lane), implying a reduction to homozygosity in tumor cells (see text).

**Fig. 1.** Immunostaining of RB protein in human prostate tissue and tumors. The specificity of peroxidase staining was verified in duplicate sections without primary antibody. (a) Benign prostate tissue (methyl green counterstain). (b) Tumor 1 (no counterstain). Peroxidase staining was performed in the presence of 0.02% cobalt chloride (21) as an intensifier. Some stromal nuclei are visible. (c) Tumor 4 (no counterstain). Endothelial cells in a small blood vessel are also stained (arrow). (d) Tumor 7 (methyl green counterstain). Tumor nuclei (green) have no peroxidase staining. Microvascular endothelial cells are identified (arrow). (x100.)
small PCR fragment suggested a deletion of about 100 bp between the two amplification primers in template DNA; its variable relative quantity in the two template samples further suggested that the small fragment was specific to tumor cell DNA and not to somatic DNA.

We suspected that tumor cells were homozygous rather than heterozygous for the putative promoter deletion because, in one portion of the specimen, the quantity of the deletion fragment vastly exceeded that of the normal fragment. If so, the other, normal RB allele must have been lost during tumorigenesis. To support this hypothesis, tumor 7 DNA was tested for loss of heterozygosity of genetic polymorphisms within RB. A polymorphism in intron 20 (24) was particularly useful for this purpose because it consisted of a variable number of short (4–5 bp) tandemly repeated sequences that could be amplified by PCR (Fig. 2B). Heterozygosity for this variable number of tandem repeats (VNTR) polymorphism was clearly demonstrated in the DNA sample derived from mostly nonneoplastic cells (Fig. 2B, left lane). However, the sample with predominance of tumor DNA showed a marked reduction in intensity of the smaller VNTR allele relative to the larger one (Fig. 2B, right lane). These results strongly suggested that one of the two RB alleles present in somatic cells from this patient was lost from tumor cells, leaving only the mutated RB allele.

Deletion within the RB promoter region was confirmed by directly sequencing the smaller DNA fragment amplified by promoter primers from tumor 7 DNA. Alignment with the normal promoter sequence (12) showed a simple deletion of nucleotides 29–131 (103 bp) (Fig. 3). This deletion removed putative transcriptional start sites at nucleotides 44 and 51, and most of a region, nucleotides 13–83, shown to be necessary for RB promoter activity (12). The translational start codon at nucleotide 277 was not involved in this mutation. Therefore, it was plausible that this deletion might inactivate RB by severely decreasing or eliminating the transcription of RB mRNA in tumor cells.

**Internal Deletion Abrogated RB Promoter Activity.** The post-mortem origin of tumor 7 precluded direct Northern blot analysis of RB mRNA. Therefore, its promoter deletion was functionally analyzed in a CAT expression system. The amplified deletion fragment (319 bp not counting linkers) was ligated upstream of the CAT gene in a construct analogous to those with normal RB promoter fragments (12). Transfection of CV-1 cells with DNA from several independent clones of this plasmid resulted in CAT activities at about the same level as that of a promoterless CAT construct (Fig. 4); normal RB promoter-containing constructs yielded CAT activities >10-fold higher than basal activity, as shown (12). These results strongly suggested that the 103-bp deletion alone was sufficient to eliminate promoter activity and thereby inactivate RB.

**DISCUSSION**

**Loss of RB Protein Expression in Human Prostate Carcinomas.** A combination of immunoblot analysis and immunostaining of cryosections was used to screen native prostate carcinoma specimens for reduced amounts or abnormal sizes of endogenous RB proteins, which suggest the presence of mutated RB alleles. Reduced or absent pp110RB expression in three tumors corresponded to reduced fractions of immunostained nuclei in histologic sections (Table 1). For tumor 5, most of the positively staining (RB+) nuclei were tumor nuclei by morphology and by the fact that the specimen consisted of >90% tumor cells. In an immuno histochemical study of RB protein expression in primary breast carcinomas, Varley et al. (32) also observed positively and negatively staining tumor cells in close proximity. Unstained tumor cells were interpreted as indicating a possible role for RB inactivation in breast tumor progression. However, it is not certain what kind of genetic mechanisms could generate such microheterogeneity. By assuming that RB alleles are similar in adjacent tumor cells, RB protein expression in tumor 5 was conservatively interpreted as normal because at least some tumor nuclei were clearly RB+. The origin or significance of such partial staining is unknown.

On the other hand, well-defined tumor cell nuclei were invariably unstained in tumor 7, whereas adjacent endothelial cell nuclei were RB+. The presence of positively staining, internal control cells strongly suggested that the lack of RB staining was actually due to RB mutation, as was indeed demonstrated. Therefore, correct identification of primary prostate carcinomas with RB mutations may be possible by concurrent detection of RB+ nuclei in prostatic epithelial or stromal cells. For tumor 6, neither pp110RB nor aberrant RB polypeptides were detected by immunoblot analysis, and distinct nuclear staining was seen in a very small fraction (less than 5%) of cells. Because this specimen was from a metastatic tumor to bone marrow, RB+ cells may represent residual bone marrow elements such as histiocytes, and tumor cells could be RB+. A detailed mutational analysis is required to confirm this proposition.
RB Inactivation by a Deletion Within Its Promoter. RB expression in one prostate tumor was ablated by two mutations: a 103-bp deletion within the promoter region of one RB allele, and loss of the second (normal) RB allele. These results support a study (12) localizing essential promoter elements to bp 13–83. Because bp 13–28 are evidently insufficient to confer promoter activity, the essential region may be further restricted to bp 29–83. The frequency of promoter mutation as a basis for RB inactivation is unknown. Several independent β-thalassemia mutations consist of base substitutions in highly conserved promoter sequences, but transcription of the β-globin gene is reduced rather than eliminated entirely (33). If reduced expression of RB is still sufficient for normal function, then deletion will be the principal promoter mutation observed in tumor cells. PCR amplification and sequencing of the RB promoter region have been hampered by its high content of C–G nucleotides (12); both procedures were found to be facilitated by the addition of dimethyl sulfoxide to reaction mixes (34). Analysis of other natural promoter mutations may further specify critical promoter sequences within bp 29–83.

Thus with our previous study of three prostate carcinoma cell lines (11), RB mutations have been characterized in 2 of 10 independent prostate cancers. The prevalence of RB mutations in these tumors may be preliminarily estimated at 10–30%. Replacement of RB protein suppressed the tumorigenic properties of DU145 prostate carcinoma cells (11), suggesting that RB inactivation plays a significant role in the formation of a subset of prostate carcinomas. It is unknown whether prostate cancers with RB mutations have different behaviors or prognoses than those with normal RB alleles. Inactivation of RB in a subset of common adult neoplasias like breast or prostate carcinomas implies the existence of several genetic pathways to neoplasia, some involving RB inactivation and some not. All pathways are likely to involve multiple additional tumor-suppressing and/or tumor-promoting genes acting in various combinations. The development of reliable methods for assessing the status of tumor suppressor gene products in clinical specimens should lead to a greater understanding of these pathways.

We thank Y.-J. Bignon for PCR reagents; N.-P. Wang and J. Wang for antibody reagents; J. Piscitelli, D. Landy, P. Scully, and P. Highgighi for histological consultation; and A. Diaz of the University of California at San Diego Cancer Center for tumor specimens. This research was supported by grants from the National Institutes of Health (EB-05758, EY-07109, CA-51495, and CA-44968), the March of Dimes (6-502), and the American Cancer Society (IN-93R). R.B. is a recipient of Physician Scientist Award EY-00278 from the National Eye Institute.