Altered Structure and Expression of the Human Retinoblastoma Susceptibility Gene in Small Cell Lung Cancer


Department of Cellular and Structural Biology, University of Texas Health Sciences Center, San Antonio, Texas 78284 [C. H. H., C.-L. H., S. L. N., A. Y. S.]; the NCI-Naval Oncology Branch, Bethesda, Maryland 20814 [A. F. G., B. E. J.]; and the Department of Pathology, School of Medicine, University of California at San Diego, La Jolla, California 92093 [W.-H. L., E. Y-H. P. L.]

ABSTRACT

Karyotypic and molecular genetic evidence has indicated that deletion or rearrangement of both chromosomes 3 and 13 may be important in the pathology of human small cell lung cancer (SCLC). The retinoblastoma susceptibility gene, RB, on chromosome 13 band q14, has previously been shown to be altered in SCLC [J. W. Harbour et al., Science (Wash. DC), 241: 353-357, 1988; J. Yokota et al., Oncogene, 3: 471-475, 1988]. Our studies of 26 SCLC tumor and normal DNA samples indicate that 6 of 6 patients whose normal cell DNA was heterozygous for an RB restriction fragment length polymorphism have lost one of the two alleles in their tumor DNA. Consistent with other studies, we find 2 of 26 tumors with homozygous deletions within the RB gene. Of 13 SCLC cell lines examined, only 3 expressed greater than trace amounts of RB mRNA. RB protein was detected in 2 of 14 SCLC cell lines examined, unlike the results of Yokota et al. (Oncogene, 3: 471-475, 1988) which showed no RB protein in any of the 9 cell lines they examined. Only unphosphorylated RB protein was detected in SCLC cell line H209, suggesting that the RB protein may be inactivated by a novel mechanism in this cell line. These data suggest that inactivation of the RB gene is a frequent if not universal event in SCLC.

INTRODUCTION

The notion that loss of specific gene function is important in the etiology of certain cancers has gained much experimental support in recent years. Cytogenetic rearrangements observed in many types of tumors, such as SCLC (1-6), renal cell carcinoma (7, 8), retinoblastoma (9, 13), and Wilms' tumor (14-17), are often accompanied by deletion of DNA sequences from specific chromosomal regions. One specifically deleted region, chromosome band 13q14, contains the retinoblastoma susceptibility gene (18) which has recently been cloned (19, 20). The RB gene has been termed a tumor suppressor gene since it is present in normal cells and deleted in RB and some other tumors. The tumor suppressing activity of the RB gene has been demonstrated recently by direct replacement of a gene encoding a normal RB protein into retinoblastoma and osteosarcoma cells in culture (22). Although no tumor suppressor gene specific for SCLC has yet been isolated, such a gene is thought to exist on chromosome 3p. Alleles of chromosome 3p markers are lost in virtually all cases of SCLC (3-6), indicating that loss of a gene(s) on 3p may be critical to the genesis of SCLC. In addition to the 3p deletion seen, many cases of SCLC show deletion or chromosomal rearrangements involving chromosome 13 (1, 2, 5, 23-25). Since chromosome 13q14 is the location of the RB gene, the loss of heterozygosity of chromosomal 13 markers raised the possibility of an involvement of the RB gene in SCLC. In agreement with the data of Harbour et al. (23), and Yokota et al. (24) our data derived from Southern and Northern hybridization studies indicate that the RB gene is frequently hemizygous and in some cases has undergone homozygous deletion in SCLC, and that RB mRNA is often not expressed in SCLC. Subtle alterations such as small deletions or rearrangements or point mutations or changes in posttranslational processing could inactivate the RB protein but would not always be detected by Southern or Northern hybridization. Thus we also chose to examine directly the RB gene product, pp110-114RB, as the most sensitive method to identify RB gene inactivation. Whereas multiple forms of RB protein can be found in most cells (26-28), only 2 of the 14 SCLC cell lines we examined expressed detectable RB protein. In line H209, the RB protein is unphosphorylated. These results differ from those of Yokota et al. (24) who did not find RB protein in any of the 9 SCLC lines they examined. The two cell lines which produce RB proteins may contain interesting mutations which could yield insight into the functional domains of the RB protein.

MATERIALS AND METHODS

Cell Lines. The SCLC cell lines used in this study were NCI-H69, H82, H128, H146, H187, H209, N417e, H526, H592, H748, H792, H930, H1184, and H1284. Nine of these lines, H69, H82, H187, H209, N417e, H526, H748, H1184, and H1284, have been examined by Harbour et al. (23) for DNA and RNA aberrations only. HBL-100, a normal human breast epithelial line, was used as a positive control for RB mRNA expression. For protein work the human neuroblastoma cell line LAN-5, kindly provided by R. Seeger, or the leukemia cell line Molt-4, were used as positive controls for pp110-114RB.

Southern Analysis. DNA was extracted from tumor tissue, normal tissue, or cell lines as described previously (4). Samples were digested with the appropriate restriction endonuclease by using conditions recommended by the supplier, and the resulting fragments were separated by electrophoresis through 0.8% agarose gels in 40 mM Tris-acetate/2 mM EDTA buffer, pH 7.2. Southern transfer to Nytran membranes and hybridization were by standard methods (29).

Northern Analysis. RNA was extracted from cell lines by the method of Chomczynski and Sacchi (30). The samples were denatured with glyoxal (31) and separated by electrophoresis on 1.2% agarose gels. RNA was transferred to Nytran blotting membrane by capillary blotting. Probes were hybridized as described below. Rat β-actin and human glutathione peroxidase cDNA clones were used as controls to verify the amount of RNA loaded in the lanes.

Probes. The RB cDNA clone pRB4.5 (19) consists of EcoRI fragments of 3.8 and 0.8 kilobases. The 3.8-kilobase EcoRI fragment was used for hybridization experiments. The plasmid p6NR0.5 (32) contains a genomic fragment from intron 1 of the RB gene. This probe detects alleles of 2.2 and 4.5 kilobases in BamHI digests of genomic DNA. The human glutathione peroxidase probes, provided by Dr. Guy Mullenbach, and the rat β-actin probe (33) were used as controls in Northern hybridization experiments. pE8 and p9A7, from Dr. Web Cavenee, are plasmids which detect restriction fragment length poly-
morpisms at 13q22-q31 and 13q33-pter, respectively (34). RB cDNA probes were hybridized according to the conditions of Lee et al. (19). Other probes were hybridized as previously reported (3).

Protein Analysis. Approximately 2 x 10⁸ cells were labeled with either [³²P]orthophosphate or [³⁵S]methionine. Briefly, cells were washed in phosphate-free or methionine-free Dulbecco's minimal essential medium, suspended in 1 ml of the same medium containing 10% dialyzed fetal calf serum, and [³²P]methionine (300 μCi/ml) or [³⁵S]methionine (250 μCi/ml) was added 30 min later. Cells were further incubated for 3 h. Cell extracts were prepared in lysis buffer containing 25 mM Tris-HCl (pH 7.4), 0.2% SDS, 50 mM NaCl, 0.2% Nonidet P-40, 0.5% deoxycholate, 200 units/ml of aprotinin, and 2 mM phenylmethylsulfonyl fluoride. Immunoprecipitation was carried out as described (26) by using polyclonal anti-RB antibodies raised against a v-erb-B fusion protein. As an alternative method, cells were lysed in SDS sample buffer, their proteins were separated on 7% polyacrylamide gels (35), and were transferred to nitrocellulose. The monoclonal anti-RB antisera was used to detect proteins by the Western blot technique (36). Antibodies to esterase D were used as positive controls.

Dephosphorylation of RB Proteins. Approximately 1 x 10⁷ cultured Molt-4 (a leukemia cell line expressing a high level of RB protein), H209, and H592 were labeled with [³⁵S]methionine (500 μCi/ml, 1134 Ci/mmolel). Cell lysates were prepared and immunoprecipitated with polyclonal anti-RB IgG as described previously (26). The protein A beads carrying immunocomplexes in 1 ml of lysis buffer were divided into two parts; 250 μl were dissociated directly in SDS sample buffer; 750 μl were washed with potato acid phosphatase reaction buffer (20 mM morpholinethesulfonic acid, pH 5.5/100 mM NaCl/1 mM MgCl₂/50 μM leupeptine) and then were incubated with 2 units of potato acid phosphatase (Boehringer) for 60 min at 37°C. After reaction, RB protein-IgG complexes were dissociated in SDS sample buffer and analyzed by 7.5% SDS-polyacrylamide gel electrophoresis. For Western blotting, unlabeled cells were used for preparing cell extracts, and monoclonal antibody PM245 (37) was used to detect the RB protein.

RESULTS

Allele Loss of Chromosome 13 Markers in SCLC. The normal tissue DNA and tumor tissue DNA from 26 individuals (24 fresh tumor samples and 2 SCLC cell lines) were examined for allele loss of three chromosome 13 polymorphisms, one of which was at the RB locus. The most distal marker p9A7 (D13S34) at 13q33-pter lost an alleie in 6 of 15 informative cases, whereas pIE8 (D13S34) at 13q22-q31 lost an alleie in 3 of 11 informative cases. A restriction fragment length polymorphism within the RB gene, detected by probe p6NR0.5, shows BsmHI alleles of 4.5 and 2.2 kilobases in heterozygous individuals (32). As shown in Table 1, in 6 of 6 cases where DNA from normal tissue was heterozygous, one allele was lost in the same individual's tumor sample. In 5 of 6 cases showing allele loss, the 4.5-kilobase BsmHI fragment was lost from the tumor. These results suggest that a gradient of alleie loss is found on chromosome 13 in SCLC with the peak of loss occurring at the RB locus. In addition, probe p6NR0.5 detected allele loss in SCLC cell line H209, a line which previously showed no RB gene aberrations (23). In SCLC 6, the two proximal markers, RB and pIE8, both lost an alleie, while the distal marker p9A7 did not lose an alleie. This suggests that in SCLC 6 loss of the RB gene was not due to the loss of an entire chromosome 13, but rather to either mitotic recombination or loss of a portion of the chromosome.

DNA Rearrangement within the RB Gene. The presence of homozygous deletions of a gene in tumor tissue is highly suggestive of an involvement in tumorigenesis and is a characteristic that was used in the isolation of the RB gene (19-21). HindIII digests of DNA samples from 12 tumors and 14 SCLC cell lines were probed with the 3.8-kilobase EcoRI fragment from the RB cDNA clone pRB4.5. Tumor DNA from patient 4 is hemizygous for the RB gene since the intensity of the majority of bands, including the 10-, 6.2-, 5.5-, 5.3-, and 2.1-kilobase fragments decreased to one-half that seen in DNA from normal tissue from the same patient (Fig. 1A, Lanes 1 and 2, respectively). Furthermore, the 7.5-kilobase fragment is absent in the remaining alleie. These results suggest that the changes in the RB gene occurred in the somatic tissue since the DNA pattern from normal tissue is unaltered. No gross abnormality was detected in the other tumor DNA samples. However, cell line H865 (Fig. 1B, Lane 4) lost the 10.0-kilobase HindIII fragment and a new band of 3.2 kilobases is present, indicating a rearrangement of the RB gene. Both of these deletions represent new findings, since DNA from neither SCLC-4 nor from cell line H865 had previously been characterized for RB gene alterations (23).

Expression of RB mRNA in SCLC. Northern blots containing RNA from 13 SCLC cell lines were probed with a 3.8-kilobase EcoRI fragment from an RB cDNA probe, pRB4.5. As seen in Fig. 1, lines H209 (Lane 5), H930 (Lane 11), and H1184 (Lane 12) express high levels of RB mRNA of the normal size. H592 expresses a lower amount of RB mRNA. Only trace or undetectable levels of the RB mRNA were apparent in the remaining cell lines. Line H748 contains a translocation of chromosome 13 that has a breakpoint in the region of the RB gene [der(20)(13;20)(q14;p13)]. H748 and line H792 (Lanes 8 and 9) were established from the same patient during different stages of his disease. These two lines do not express detectable levels of RB mRNA, although no DNA alteration was seen by Southern hybridization. The RB mRNA expression data are in close agreement with those of Harbour et al. (23), except that we detected a trace of RNA in line H526, while Harbour et al. did not detect RB RNA in this cell line.

Expression of pp110-114⁸⁸. Protein from 14 SCLC cell lines was labeled metabolically with [³²P] or [³⁵S]methionine and immunoprecipitated with antibodies directed against the RB protein (26). In cells labeled with orthophosphate, only H592 showed a high level of phosphorylated pp110-114⁸⁸ (Fig. 3). RB proteins were detected both in H592 and in H209, as well as in the control cell line Molt-4, in extracts of [³⁵S]methionine labeled cells (Fig. 4A, Lanes 1a, 2a, and 3a). Multiple forms of RB protein were found in Molt-4 and H592 (Lanes 1a and 2a). However, only the fastest moving band was detected in H209 (Land 3a). After treatment of the immunoprecipitates with potato acid phosphatase, only the most rapidly migrating band was seen in extracts from Molt-4 and H592 (Lanes 1b and 2b). In H209, phosphatase treatment did not change the mobility of the RB protein. Taking together the absence of [³²P]-labeled RB protein in H209 and the similar mobility of the RB protein both before and after phosphatase treatment, we conclude that the RB protein expressed in H209 is not phosphorylated. Some additional proteins appeared to coprecipitate with the RB protein in certain cells, i.e., several additional bands were found in

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* Number of individuals showing alleie loss/total number of individuals heterozygous for the marker.

J. Whang-Peng, personal communication.
RB aberrations in SCLC

Fig. 1. Homozygous deletion within the RB gene in SCLC. HindIII-digested DNA from SCLC tumors, normal tissue, or SCLC cell lines were probed with the 3.8-kilobase (kb) EcoRI fragment from the RB cDNA. A. DNA from tumor (Lanes 1, 3, and 5) and normal tissue (Lanes 2, 4, and 6) from SCLC patients 4 (Lanes 1 and 2), 5 (Lanes 3 and 4), and 6 (Lanes 5 and 6). B. DNA from SCLC cell lines H748, H774, H847, and H865 (Lanes 1 through 4, respectively).

Fig. 2. Analysis of RB RNA expression in SCLC cell lines. Ten μg of total RNA were denatured with glyoxal, separated on 1.2% agarose gels, transferred to Nytran, and hybridized to an RB-1 cDNA probe (A) or to a rat β-actin cDNA probe (B). RNA was from SCLC cell lines H69, H128, H146, H187, H209, N417C, H526, H592, H748, H792, H930, H1184, and H1284, (Lanes 1–13, respectively). The position of the normal 4.7-kilobase (kb) RB transcript and the 2.0-kilobase actin transcript are indicated.

H592, and these proteins apparently were unaffected by phosphatase treatment (Lanes 2a and 2b). The nature of these proteins needs further characterization.

Multiple forms of RB protein also were detectable with monoclonal antibody PMG245 in Western blotting analysis (Fig. 4B). All forms of RB protein could be found in Molt-4 and H592 (Fig. 4B, Lanes 1a and 2a), but only one faster moving band is found in H209 (Fig. 4B, Lane 3a). After reaction with potato acid phosphatase, a single fast moving band of presumably unphosphorylated RB protein was detected in all three cell lines (Fig. 4B, Lanes 1b, 2b, and 3b).

Discussion

Retinoblastoma survivors frequently develop secondary cancers, including osteosarcoma and soft tissue sarcomas, later in life (38, 39), indicating that the RB gene may be important in the etiology of other tumors in addition to retinoblastoma. The involvement of the RB gene in other cancers has been tested directly with the molecularly cloned RB gene. A high frequency of RB mutation has been shown in osteosarcoma (39–42), soft tissue sarcomas (40, 42), and breast cancers (43–45).

SCLC is not found as a common second site tumor in survivors of hereditary retinoblastoma (46). However, loss of heterozygosity of chromosome 13 DNA markers has been reported for SCLC (5, 23), and 9 of 9 SCLC lines examined by Yokota et al. expressed no detectable RB protein (24). A specific polymorphic probe from within the first intron of the RB gene, p6NR0.5, was used to analyze mutation of the RB gene in primary SCLC tumors, and in all 6 informative cases, tumor cells lost an allele of the RB gene (Table 1). Genomic DNA analysis with a RB cDNA probe demonstrated a homozygous
Fig. 3. Immunoprecipitation of [32P]-labeled pp110-114RB* from SCLC cell lines. Proteins were labeled with [32P] as described in "Materials and Methods" and immunoprecipitated with anti-pp110 antisera. Lanes contained immunoprecipitates from LAN-1 cells (Lane 1) or SCLC cell lines NCI-H69, H82, H128, H146, H187, H209, N417E, H526, H592, H748, H792, H930, H1184, and H1284 (Lanes 2–15).

Fig. 4. Dephosphorylation analysis of the RB protein. (A) Molt-4 (Lanes 1a and 1b), H592 (Lanes 2a and 2b), and H209 (Lanes 3a and 3b) were labeled with [35S]methionine. Immunocomplexes of RB proteins were either analyzed directly (Lanes a) or after treatment with potato acid phosphatase (Lanes b) as described in "Methods and Methods." The SDS-polyacrylamide gel was processed for fluorography and was exposed for 2 days on X-ray film. Brackets indicate multiple forms of phosphorylated RB protein. (B) Western blotting of cell lysates from Molt-4, H592, and H209 cell lines. Immunoprecipitation and potato acid phosphatase treatment were performed as in (A) but on unlabeled cell extracts. RB proteins were detected with monoclonal antibody PMG245. Triangles indicate the single band of RB protein found in H209. Ordinates, molecular weights in thousands.

deletion of the 7.5-kilobase HindIII fragment in one tumor tissue sample. In line H865, homozygous deletion of a 10-kilobase HindIII fragment and the appearance of a novel 3.2-kilobase fragment was observed. The 10-kilobase HindIII fragment contains exons 20 through 23, which includes part of the RB protein coding region (47, 48). The rate of homozygous deletion within the RB gene (2 of 26) is comparable to that found by Harbour et al., using the same probe (3 of 22 cell lines showed 3' rearrangements). However, the samples in which we detected homozygous deletions were not studied by Harbour et al., who found normal RB mRNA in to those of Yokota et al. (24), who found normal RB mRNA in the majority of SCLC cell lines. Among 14 lines tested, only 1, H592, had the normal pattern of RB proteins, including phosphorylated and unphosphorylated forms (Fig. 3), and 1, H209, produced unphosphorylated RB protein (Fig. 4). No protein can be identified in H930 and H1184 with either 32P or [35S]methionine labeling. As these two lines express RB proteins in SV40 or adenovirus transformed cells (37, 53), multiple forms of phosphorylated RB proteins are present. Dephosphorylation occurs when cells leave S phase and maximum phosphorylation can be detected again at M phase (49–52). To rule out the possibility that the predominant unphosphorylated RB protein in H209 is due to slow growth of the cells, we have treated the cells with Nocodazole to increase the population of the cells at M phase, or used a thymidine block to increase the population of cells in S phase. Again, only the fast moving form of RB protein was detected (data not shown). Therefore it is plausible that a mutation affecting the phosphorylation of the RB protein has occurred in H209.

Recent studies have demonstrated that the RB protein is phosphorylated primarily on serine and threonine residues (27, 28). Many consensus kinase phosphorylation sites can be identified within the RB protein, including six sites for the cdc2 kinase. In vitro experiments demonstrated that cdc2 was able to phosphorylate the RB protein at specific sites. Further characterization will reveal the nature of the RB gene defect which results in lack of RB protein phosphorylation in H209. Multiple forms of pp110-114RB* proteins can be found in most normal cells (26–28). The band with the greatest mobility represents the unphosphorylated form and the three more slowly migrating bands are multiple forms of the phosphorylated RB proteins (27, 28). Differences in the biochemical properties of phosphorylated and unphosphorylated RB proteins have been shown recently. Although RB proteins have been shown to bind to SV40 large T-antigen and adenovirus E1A proteins in SV40 or adenovirus transformed cells (37, 53),
association of the RB protein with large T-antigen was limited to the unphosphorylated form (27). Whether or not the DNA binding or E1A protein binding activities associated with the RB proteins is affected by phosphorylation is unknown. However, the modulation of sequence-specific DNA binding of the heat shock factors by phosphorylation has been proposed recently (54). Phosphorylation is a widely occurring reversible modification used in the regulation of many biological processes (55), and further characterization of the unphosphorylated RB protein product in H209 should help to clarify the significance of RB phosphorylation.

Our data support the hypothesis (23, 24) that inactivation of the RB gene is a frequent event in SCLC. Unlike retinoblastoma, SCLC does not have a clear hereditary pattern. It is likely that in SCLC mutation of both RB alleles occurs in the somatic cells, as in sporadic retinoblastoma. The absence of RB gene rearrangements as detected by Southern hybridization analysis of normal cell DNA from 100 SCLC patients using the 3.8-kilobase 3' RB probe supports this conclusion (data not shown). Previous studies indicated that the short arm of chromosome 3 is deleted in virtually all cases of SCLC and suggest that this region may harbor another tumor suppressor gene analogous to the RB gene. Whether mutations of both RB and a chromosome 3p gene are required for SCLC pathogenesis or whether one or both of the changes is progressive cannot be distinguished with the current data. However, the frequency of alteration of the RB gene in SCLC suggests that it has an important role in this disease. Studies to insert human chromosome 3 into SCLC cells and transfection of SCLC with a wild type RB gene are currently under way to address their individual roles in the genesis of SCLC.

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