Absence of Retinoblastoma Protein Expression in Primary Non-Small Cell Lung Carcinomas

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Abstract

Retinoblastoma (RB) protein expression was examined in paraffin and frozen tissue sections of 36 primary non-small cell lung carcinomas (NSCLC) using immunohistochemistry with confirmation by direct Western blotting. A normal RB protein staining pattern was present in 24 and absent in 10 NSCLC. Two additional RB positive primary tumors have been identified in archival paraffin-embedded tissue sections, which provides a practical means to examine the status of the RB protein in individual tumor cells by immunohistochemistry. A RB protein immunohistochemical staining method has been described previously. According to our initial findings, normal and known RB+ tumor cells either in culture or in vivo display a cell-by-cell heterogeneous RB staining pattern with variable proportions of cells having unstained nuclei, since not only the phosphorylation state but also the cellular concentration of the RB protein change significantly during the cell cycle (16). In fact, cells in the middle G1 or G0 phase of the cell cycle have no detectable nuclear staining, although they have a normal functional RB gene (16). Nevertheless, in general, RB+ and RB− staining tumors were still distinguishable from each other (16). In the present study, we used the same reagent and criteria to document whether there was altered RB expression in primary or metastatic NSCLC. Direct Western and Northern blot analyses of the RB gene products were also done on tumor samples when frozen tissue materials were available for further confirmation of the immunohistochemical studies. We also report for the first time that excellent RB staining can be attained using the RB-WL-1 anti-RB polyclonal antibody (4) in either new or archival paraffin-embedded tissue sections, which provides a practical means to examine the RB protein status in various human cancers as a potential prognostic variable.

Introduction

The RB susceptibility gene is a prototypical tumor suppressor gene, the genetic basis of which was initially deduced from patients with the hereditary form of retinoblastoma (1,2). This gene encodes a 110-116-kDa nuclear protein with cyclical changes in phosphorylation (3–7). There is also growing evidence suggesting that the loss of RB gene function is a key factor in the initiation and/or progression of a diverse group of human cancers, including those of the lung, breast, bladder, and prostate (8–13). In addition, the RB gene product may be an important prognostic variable in patients with high-grade human sarcomas (14). One of the first studies of RB gene changes in tumors usually not associated with the development of retinoblastoma was that of small cell lung carcinoma. Structural changes in the RB gene and the absence of RB gene expression at either the RNA or protein level were frequently seen in small cell lung carcinoma cell lines, which provided the basis for concluding that the loss of RB function is important in the development of this tumor type (8, 9). A few NSCLC cell lines were also found to have no RB expression (8, 9). However, the role of the RB gene in NSCLC, especially in primary tumors, has not been determined. It is recognized that the difficulty in documenting functional loss of the RB gene in primary tumor could reflect the fact that there is an abundance of normal stromal tissue in many tumor specimens. One might anticipate, therefore, that the most straightforward and sensitive approach to identifying primary tumors which have lost RB function as well as determining whether such loss is related to initiation versus progression of a given tumor would be to examine the status of the RB protein in individual tumor cells by immunohistochemistry (15). A RB protein immunohistochemical staining method has been described previously. According to our initial findings, normal and known RB+ tumor cells either in culture or in vivo display a cell-by-cell heterogeneous RB staining pattern with variable proportions of cells having unstained nuclei, since not only the phosphorylation state but also the cellular concentration of the RB protein change significantly during the cell cycle (16). In fact, cells in the middle G1 or G0 phase of the cell cycle have no detectable nuclear staining, although they have a normal functional RB gene (16). Nevertheless, in general, RB+ and RB− staining tumors were still distinguishable from each other (16). In the present study, we used the same reagent and criteria to document whether there was altered RB expression in primary or metastatic NSCLC. Direct Western and Northern blot analyses of the RB gene products were also done on tumor samples when frozen tissue materials were available for further confirmation of the immunohistochemical studies. We also report for the first time that excellent RB staining can be attained using the RB-WL-1 anti-RB polyclonal antibody (4) in either new or archival paraffin-embedded tissue sections, which provides a practical means to examine the RB protein status in various human cancers as a potential prognostic variable.

Materials and Methods

Tumor Specimens and Cell Lines. All the fresh tumor tissues, two NSCLC cell lines, and paraffin tissue sections were obtained from the Methodist Hospital (Houston, TX) and the Denver General Hospital (Denver, CO). Fresh tumor tissues were immediately frozen in liquid nitrogen or dry ice and stored for varying periods at −80°C. Frozen tissue sections of each specimen were formalin fixed and stained with hematoxylin–eosin. Both frozen and paraffin sections were examined microscopically to confirm the histological diagnosis and the abundance of tumor cells. Tumor staging was done according to the new tumor-node-metastasis staging system of the American Joint Committee on Cancer based on tumor size, lymph node metastases, and distant metastases (17). The staging information on each tumor was revealed only after all experimental analyses were completed.

Immunohistochemistry. The highly specific affinity-purified polyclonal anti-RB antibody, RB-WL-1, was raised by immunization of rabbits with a synthetic peptide derived from exon 10 of the human RB gene (4). This antibody recognizes both the unphosphorylated and phosphorylated RB protein by immunoprecipitation, Western immunoblotting, and immunohistochemical staining (4, 16). The RB staining using this antibody can be completely blocked in the presence of an excess of the immunizing RB peptide (16). A preimmune serum from the same rabbit was used in all experiments as a negative control. Immunohistochemical analysis was performed on either dewaxed paraffin sections or paraformaldehyde-fixed frozen tissue sections as previously described (16). Briefly, the sections were washed in phosphate-buffered saline followed by preincubation with 1.5% normal goat serum in phosphate buffer within a moist chamber for 4 h at room temperature. Those sections were then incubated overnight with RB-WL-1...
antibody at a final concentration of 2 μg/mL. After being washed with 6 changes of phosphate-buffered saline containing 0.02% Triton X-100 over 15 min, the slides were processed for immunostaining with the avidin-biotinylated peroxidase complex method (Vector Laboratories, Burlingame, CA) according to the technical manual. The tissue sections were briefly counterstained with Mayer’s hematoxylin before mounting. Cultured cells were grown on sterile coverslips in tissue culture dishes overnight, fixed with 45% acetone/10% formaldehyde in 0.1 M phosphate buffer for 5 min, and then processed for immunohistochemical assay as described above.

A tumor was considered to be RB+ if any of its malignant cells had RB nuclear staining, whereas a tumor was defined as RB− only if all of the cancer cells had no RB nuclear staining. Altered RB protein expression is defined by lack of RB protein staining in every tumor cell of (a) the primary tumor, (b) a major focal area of the primary tumor, or (c) the corresponding metastatic tumor(s).

Western Immunoblots. Cell lysates were prepared from tumor tissues or exponentially growing cell cultures and processed for direct Western immunoblotting as described (16). Each lane was loaded with 50 μl of the cell lysate containing 80 μg of total cellular proteins.

mRNA Isolation and Northern Blots. Polyadenylated RNAs were isolated from 0.1–0.4 g of tumor tissue of each specimen using a FastTrack mRNA isolation kit (Invitrogen). The mRNAs (2 μg/lane) were denatured using the glyoxal and dimethyl sulfoxide method (18), electrophoresed in 1% agarose gel in 10 mM phosphate buffer (pH 6.5–7.0), and transferred to nylon membranes (Hybond-N; Amersham). The membranes were then hybridized with mixed 32P-labeled 3.8-kilobase RB probe and 2.0-kilobase β-actin probe.

Results and Discussion

Altered Expression of the RB Gene in Primary and Metastatic NSCLC Determined by Immunohistochemistry. In a pilot study, we examined the RB protein expression in two NSCLC cell lines, pTC2 and COLO759 (Fig. 1, A and B). The exponentially growing pTC2 cell culture showed a normal, heterogeneous nuclear staining pattern of the RB protein, while no RB protein expression was detected in any COLO759 cells. This latter cell line was initially established from a primary poorly differentiated adenocarcinoma of the lung. To rule out the possibility of tissue culture artifacts, frozen and paraffin tissue sections corresponding to different parts of the primary tumor specimen were also examined by cell staining. As shown in Fig. 1D, no RB-positive tumor cells were identified in any of these sections, although some proliferating stromal cells were found to be RB+, which in turn served as an internal control for the staining reactions. These results suggested that altered RB protein expression likely had an etiological role in the formation of this specific tumor, since all primary tumor cells had lost RB function. In addition, it confirmed that the RB loss in COLO759 cells was not a tissue culture artifact.

The study was then expanded to analyze RB protein expression in a total of 36 primary NSCLC by immunohistochemical staining of paraffin-embedded tissue sections. In addition, frozen tissue sections, whenever they were available, were also stained to provide a comparison between the two techniques. Twenty-four tumors (67%) had by definition a normal, cell-by-cell heterogeneous RB+ staining pattern. The ratio of the tumor cells with stained versus unstained nuclei, as well as the staining intensity of individual RB positively stained tumor cells, were variable even within the same tumor or among RB+ tumors with the same histological subtype. Nevertheless, all such cases were defined as RB+ tumors. For example, as illustrated in Fig. 1, C and E, two squamous carcinomas of the lung are shown which have intensive RB+ staining. In contrast, 10 primary tumors (28%), including the case mentioned above (Fig. 1D), were RB−, since they lacked RB protein expression in every tumor cell. This indicated to us that RB functional loss is a frequent occurrence in NSCLC. In addition, we found that two primary RB+ NSCLC, one a squamous carcinoma and the other an adenocarcinoma, had major focal areas in which all tumor cells were missing RB protein. As shown in Fig. 1, G and H, respectively, the major RB+ and RB− areas of the primary adenocarcinoma were easily distinguished from each other in their staining patterns. Moreover, the portion of tumor in which homogeneous RB− cell staining was identified seemed to be poorly differentiated with more mitotic figures. These findings would indicate that although altered RB expression was not involved in the initiation of these two tumors, it may have been important in their progression.

Corresponding lymph node metastases and/or distant metastases were available for 5 of the 36 primary tumors. Metastatic lesions from two RB+ and two RB− primary tumors had the same RB staining patterns as their primary tumors. However, the fifth metastasis was found to be RB− (Fig. 1F), although the corresponding primary tumor showed a high level of RB expression (Fig. 1E). Again this suggested that functional loss of the RB gene could also have an important role in the progression of certain NSCLC.

Finally, it is of practical importance to note that the results of immunohistochemical evaluation obtained from either paraffin or frozen tissue sections were consistent. As shown in Fig. 1, in fact, the quality of cell staining using paraffin sections cut from 10-year-old tissue blocks (Fig. 1E) was often as good and in certain cases better than that obtained from frozen tissue sections (Fig. 1C). Twenty-four of 36 cases had both frozen and paraffin sections available for comparison, and the staining patterns were always consistent with each other.

Altered Expression of the RB Gene in Primary NSCLC Determined by Direct Western and RNA Blotting. The results of quantitation of total cellular RB protein in primary tumors by direct Western blotting confirmed our immunohistochemical analyses. Direct Western blotting was performed on the RB+ pTC2 and RB− COLO759 lung cancer cell lines, on 19 of the 36 primary NSCLC where fresh tumor tissues were available, and on a normal adjacent lung specimen. p110RB was detected only at a low threshold level in normal lung tissues (Fig. 2B, Lane 19). Based on equal loadings of total cellular proteins, both the RB+ cell line (Fig. 2A, Lane 2) and the primary tumor (Fig. 2B, Lane 2) from which the cell line was established showed a normal and intense RB protein banding pattern. In contrast, no RB protein was detected in the RB− tumor as shown in Fig. 1D (Fig. 2B, Lane 3) or in the cell line, COLO759, which was derived from the primary tumor (Fig. 2A, Lane 3). We also found no RB protein or only faint RB protein bands (below the threshold level of RB protein in normal lung tissues) in four primary tumor samples which previously had been determined to be RB− by immunohistochemical analysis (Fig. 2B, Lanes 5, 13, 14, and 20). All RB+ primary tumors defined by cell staining had either a strong p110RB band or increased levels of both p110RB and pp110RB (Fig. 2B, Lanes 4, 6–12, 15, 16, 18, and 21), with the exception of one tumor specimen (Fig. 2B, Lane 17), in which the tumor cells accounted for less than 5% of the tissue sections when stained with hematoxylin-eosin. As reported previously (16), normal nondividing adult tissues do not show pp110RB. Therefore, the presence of pp110RB on Western blots (e.g., see Fig. 2B, Lanes 2, 6, 7, 9–12, and 16) may actually be sufficient to define a given tumor sample as RB+.
In a parallel experiment, polyadenylated RNAs have also been isolated from 12 of the primary tumor specimens, and Northern blotting was done based on equal loadings of the mRNAs. As shown in Fig. 3, all 6 RB+ tumors examined had normal RB mRNA expression (Lanes 1–6). However, only one of the 6 RB− tumors as defined by cell staining and Western blotting lacked detectable RB mRNA (Fig. 3, Lane 8), whereas the levels of RB mRNA present in the other RB− tumors appeared to be normal (Fig. 3, Lanes 7, 9–12). Although the presence of fibrous stroma in primary tumors could increase significantly the total protein content and thus dilute the relative amount of stromal RB protein, this factor usually does not affect the ratio of stromal RB mRNA to other species of cellular mRNAs. Therefore, as expected, RNA blotting was less sensitive than immunohistochemical RB staining or direct Western blotting in identifying RB− primary tumors.

Correlation between RB Protein Status in Primary NSCLC and Their Pathological Stages. The primary NSCLC included various histological subtypes, i.e., adenocarcinoma, bronchioalveolar adenocarcinoma, large cell adenocarcinoma, and squamous carcinoma, and were randomly selected. The histological subtype of human NSCLC has not been shown to be a consistent prognostic factor, whereas the tumor stage is considered the most important factor in lung cancer prognosis. Staging is primarily based on tumor size as well as lymph node and distant metastases (17), and high-stage NSCLC usually has an unfavorable outcome.

After the RB protein status of all the individual tumors was
Fig. 2. Direct Western blotting quantitation of RB protein in NSCLC cell lines and primary tumors. Each loading was normalized by total cellular protein (80 μg/lane). A and B; Lane 1, human fibroblast W1-38, normal control; A, Lane 2, RB+ NSCLC cell line, pTC2; Lane 3, RB- NSCLC cell line, COLO759; B, Lanes 2 and 3, the RB+ and RB- primary tumors from which the cell line pTC2 and COLO759 were established, respectively; Lanes 4, 6-12, 15-18, and 21, immunohistochemically defined RB+ primary tumors; Lanes 5, 13, 14, and 20, the immunohistochemically defined RB- primary tumors. The RB+ tumor of Lane 17 contained less than 5% tumor cells. Lane 19, adjacent normal lung specimen.

Fig. 3. Northern blot analysis of RB mRNA in primary NSCLC. Polyadenylated RNAs were isolated as described in “Materials and Methods.” Northern blotting was done on the immunohistochemically defined RB+ (Lanes 1-6) and RB- (Lanes 7-12) primary tumors to provide a comparison. The RB gene probe detects a 4.7-kilobase transcript, while the β-actin probe detects a 2.0-kilobase transcript. Only one of the RB- primary tumors showed an absence of RB mRNA.

Table 1 Altered RB protein expression in NSCLC

<table>
<thead>
<tr>
<th>Group</th>
<th>Pathological stage</th>
<th>RB protein expression</th>
<th>Normal</th>
<th>Altered*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 23)</td>
<td>I, II</td>
<td>18</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>2 (n = 13)</td>
<td>III, IV</td>
<td>5</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

* The difference in altered RB protein expression between groups 1 and 2 was significant (P < 0.05, calculated using the χ² test).
* The altered RB protein expression means, by definition, lack of RB protein staining in every tumor cell of (a) the primary tumor, (b) a major focal area of the primary tumor, or (c) corresponding metastatic tumor(s).

ascertained, their pathological stages were revealed. As summarized in Table 1, it was found that more high-stage tumors had altered RB protein expression. The difference in occurrence of altered RB protein expression between groups 1 (stages I and II) and 2 (stages III and IV) was significant (P < 0.05). There was no difference in histological subtypes between the two groups (data not shown).

In summary, our results show a lack of RB protein expression in 10 (28%) of the primary NSCLC examined. In addition, 2 of 36 primary tumors had major foci in which all tumor cells were missing RB protein and, in a third case, loss of RB protein was documented in a metastatic lesion whereas the primary tumor was RB+. Loss of RB function in these latter three cases was not involved in the tumor initiation but likely could have been important in their progression. In contrast, since there was a total loss of RB expression in the other 10 primary tumors, we would suggest that such functional loss might be involved in the initiation of a significant proportion of NSCLC. These findings are in general consistent with the previous report indicating that 9 of 21 primary NSCLC had lost heterozygosity for a chromosome 13q DNA probe (19). In addition, a current study has found that approximately 10% of primary NSCLC have abnormal RB mRNA expression, although deletions of the RB gene as determined by Southern analysis were rare in this series of 160 patients (20). We have been able to demonstrate a high incidence of RB alterations in NSCLC by means of immunohistochemical RB protein staining, since this approach allows one to assess RB protein expression in individual tumor cells. The quantitation of total RB protein in these primary tumor specimens by direct Western immunoblotting confirmed the accuracy of our immunohistochemical findings.

Finally, the evaluation of RB protein expression in primary tumors, which we now have shown for the first time can be determined by staining paraffin tissue sections, may become an additional prognostic parameter or an important molecular marker in human NSCLC. Particularly relevant to this issue is the recent report that loss of functional RB protein is a significant negative prognostic factor in high-grade adult soft tissue sarcomas (14). Since we found that more high-stage (stages III and IV) NSCLC lack RB protein expression as compared to the low-stage (stages I and II) tumors (P < 0.05), it may also suggest that altered RB protein expression would result in a poorer prognosis for NSCLC. However, since an insufficient number of cases were examined in this study to correlate clinical outcome with RB alteration, we have not been able to determine yet whether RB functional loss is a prognostic indicator in NSCLC. Further studies to extend these initial findings are now in progress in our laboratory.

Acknowledgments

The authors acknowledge the participation of Elaine Spector, Ph.D., and the other surgeons and pathologists who provided tissues and histological preparations.
References


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