Loss of Heterozygosity of the Rb Gene Correlates with pRb Protein Expression and Associates with p53 Alteration in Human Esophageal Cancer

Eric Poe Xing, Guang-Yu Yang, Li-Dong Wang, Stephanie Tao Shi, and Chung S. Yang

Laboratory for Cancer Research, College of Pharmacy, Rutgers University, Piscataway, New Jersey 08854-8020 [E. P. X., G-Y. Y., S. T. S., C. S. Y.]; and Henan Medical University, Zhengzhou, 457500 China [L-D. W.]

ABSTRACT
To understand the alterations of Rb tumor suppressor gene and the relationship between defects in the Rb and p53 pathways in human esophageal carcinogenesis, we examined the loss of heterozygosity (LOH) of the Rb gene and immunohistochemical staining of pRb protein in 56 esophageal squamous cell carcinoma specimens and related the results to the p53 gene alterations. Using four intron-specific polymorphic markers as probes, we observed LOH of the Rb gene in 30 of the 55 informative tumor samples. Immunohistochemical analysis revealed different patterns of pRb expression among the tumor samples. In the 56 cases, 16 displayed extensive pRb staining comparable to that of the adjacent normal epithelia, whereas 33 showed either significantly decreased or no pRb staining and 7 had a focal staining pattern reflecting heterogeneous cancer nests in the tumor with respect to Rb status. In the tumor samples containing Rb LOH, 90% showed low or no pRb expression, whereas in samples without Rb LOH, only 20% had altered pRb expression. There was a strong association between LOH of the Rb gene and alteration of pRb expression in our samples (P < 0.0001), suggesting LOH is a main event leading to Rb inactivation. We found that Rb LOH was more frequent in tumors with p53 mutations (P < 0.05), which occurred in 31 of the 49 cases analyzed. When the status of Rb and p53 alterations was evaluated by the combined results of immunohistochemical and genetic analyses, we found that alteration of Rb and p53 had an even stronger association in our esophageal squamous cell carcinoma samples (P = 0.0015).

INTRODUCTION
The Rb gene, located on chromosome 13q14.2, was the first tumor suppressor gene to be identified in humans and was initially determined to be associated with the development of retinoblastoma (1). Rb encodes a cell cycle control protein that is at the convergence of several positive and negative regulatory pathways that are often referred to collectively as the Rb pathway (2). Hypophosphorylated pRb, which is regarded as the active form, can form stable complex with various transcriptional activators of the E2F family and halt the cell cycle progression during G1 (3). Suppression of pRb function through hyperphosphorylation causes the release of the E2F factors and triggers a burst of gene expressions that facilitates G1-S transition (4, 5). Functional loss of the Rb gene frequently occurs in the carcinogenic processes of many types of cancer (reviewed in Refs. 6 and 7). The mechanism leading to such loss usually involves the loss of one allele by a germ-line or early somatic alteration and a subsequent alteration of the other allele, which was first observed in retinoblastoma, and formed the basis for the two-hit model of cancer development (1, 8). Therefore, LOH4 at the Rb locus is an important event reflecting potential functional alteration in the Rb gene. LOH on 13q, where Rb is located, is a common feature in many types of cancer involving bladder, lung, breast, head and neck, and other organs (9–25). However, it is not always possible to observe concordance of LOH at 13q and loss of pRb protein expression in different types of tumor, suggesting that either the selected LOH markers are for other genes near the Rb locus or other mechanisms are involved in the inactivation of the Rb gene. In human esophageal cancer, LOH at the Rb locus was observed in 54% of SCCs and in 36% of adenocarcinomas (16, 17), but whether it leads to a decrease in pRb expression is not clear.

Recently, an association between aberrant pRb and p53 expression was observed in bladder and several other types of cancer (19–25). p53 is critical for coordinating multiple growth control checkpoints in response to genotoxic insults and abnormal proliferation (26). Wild-type p53 can block malignant cell

4 The abbreviations used are: LOH, loss of heterozygosity; SCC, squamous cell carcinoma; ESCC, esophageal SCC; VNTR, variable number of tandem repeats; SSCP, single-strand conformation polymorphism.
transformation by inhibiting proliferation, facilitating DNA repair, and stimulating apoptosis in genetically injured cells (reviewed in Ref. 27). Intuitively, an increased proliferation capacity due to Rb loss together with a decreased rate of apoptosis due to p53 alteration would greatly enhance the tumorigenic potential of the affected cells. Indeed, recent studies in the murine system showed that, although germ-line mutation in Rb or p53 resulted in predisposition to cancer (28, 29), heterozygous mice mutant for both Rb and p53 showed reduced viability and novel cancer pathology, including increased cancer burden (30). Recently, pRb was found also to have an antiapoptotic function (31). Loss of Rb can activate apoptosis at least in part via elevated p53 expression, and loss of p53 gene function prevents cell death in the central nervous system of Rb-mutated mouse embryos (32). It was observed that several types of DNA tumor viruses (i.e., SV40, papilloma viruses, adenoviruses, and so on) contain proteins that bind and inactivate both pRb and p53 (33–35), which implicates that DNA tumor virus. Viruses have developed mechanisms for attacking critical cellular antitumorigenic pathways by inactivating both pRb and p53 protein. Furthermore, it was observed that the cellular oncoprotein MDM2 could also negatively regulate both pRb and p53 through protein-protein interaction (36). Therefore, it is possible that inactivation of both Rb and p53 genes in the cell produces a synergistic effect, which imposes stronger selective pressure for cellular transformation. Indeed, it was reported that, in cells that sustained lesion in the Rb pathway, there was a strong selection for the loss or inactivation of wild-type p53 (37).

In our previous studies of human ESCC, we identified frequent intragenic mutations in the p53 gene (38, 39). Together with the previous report of Rb LOH in ESCC and due to the important roles of both Rb and p53 pathways in safeguarding normal cell proliferation, these results suggest that it is possible that both genes can be the main alteration targets during ESCC formation. In this study, we studied the relationship between LOH of the Rb gene and the altered expression of pRb in 56 ESCC patients from a high incidence area in Linzhou (formerly Linxian), China, and showed that Rb LOH is strongly associated with altered pRb expression. Furthermore, a close analysis of the correlation of Rb alterations with p53 alteration in these samples suggested an association of the Rb and p53 alterations in ESCC.

MATERIALS AND METHODS

Tissue Collection and DNA Preparation. Matched pairs of normal and tumor specimens were dissected from surgically resected esophagi of 56 primary ESCC patients in Linzhou, China. All specimens were fixed and stored in 80% ethanol before use. Serial sections (5 μm) were made from the paraffin-embedded tissue blocks for both DNA preparation and immunohistochemical studies. Tumor regions were identified by histopathological examination of a representative H&E-stained slide, and the two adjacent slides were used for dissection of tissues from the corresponding tumor regions. Normal tissues were directly dissected from the adjacent epithelium. Genomic DNA was extracted by proteinase K digestion and phenol/chloroform extraction.

PCR-based RFLP Analysis of Rb Allelic Status. The primer sets used in this study are listed in Table 1. PCRs were carried out in a reaction volume of 25 μl with 800 nm each primer, 250 μM dNTP, 1.5–2.5 mM MgCl₂, standard PCR buffer, and 1 unit of Taq polymerase (all from Life Technologies, Inc., Gaithersburg, MD). Typical reaction conditions were as follows: 5 min of denaturation at 95°C before addition of the Taq polymerase; 2 min at 95°C, 2 min at annealing temperature (Tₘ), and 2 min at 72°C for initial cycle; and then 30 cycles of 90 s at 95°C, 45 s at Tₘ, and 45 s at 72°C, followed by 2 min at 72°C. Tₘ cycle number, and Mg²⁺ were optimized for each primer set, based on pilot experiments. Normal and tumor DNA (0.1 μg; 0.5 μg) were used for PCR amplification. To generate the RFLP pattern for LOH analysis, 10 μl of PCR product were digested twice with 1 unit of appropriate restriction enzyme (BanHI for intron 1 RFLP and Xbal for intron 17 RFLP) in a total volume of 20 μl, each lasting 4 h, followed by heat inactivation of the old enzyme. The resulting mixtures were resolved on 2.5% agarose gels and stained with ethidium bromide.

PCR-based VNTR and Microsatellite Analysis of Rb Allelic Status. PCRs were performed as described in the RFLP assay. For LOH analysis of the intron 17 VNTR marker, PCR products were directly electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. For intron 20 VNTR, the forward primers were radiolabeled at 5’ end with [γ-³²P]dATP, and PCR products were resolved on 6% denaturing polyacrylamide gel and radiographed for 4 h before analyzing the signals. Semiquantitative measurements were performed for LOH determination. In brief, for both RFLP and VNTR analyses, the ratio of intensity of the lost allele versus that of the retained allele was measured and calculated using a computer imaging system (Image-Pro Plus; Media Cybernetics). A LOH was defined by the signal ratio in the tumor lane being <25% of that in the normal lane.

Table 1 Oligonucleotides for determining Rb LOH

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Locus</th>
<th>Polymorphism type</th>
<th>Product size (bp)</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>216a</td>
<td>Intron 1</td>
<td>RFLP</td>
<td>195</td>
<td>5'-CAGGCACAGGCCGGCCGGAG-3'</td>
</tr>
<tr>
<td>2356</td>
<td>Intron 17</td>
<td>BamHI</td>
<td>190</td>
<td>5'-CTGGCAAGCTCGCCCTGGT-3'</td>
</tr>
<tr>
<td>99311</td>
<td>Intron 17</td>
<td>RFLP</td>
<td>190</td>
<td>5'-TCCACCTCAGGCTCTCTTAG-3'</td>
</tr>
<tr>
<td>99500</td>
<td>Intron 17</td>
<td>XbaI</td>
<td>190</td>
<td>5'-GTAGGCCAAGAGTGGCAGCT-3'</td>
</tr>
<tr>
<td>123888</td>
<td>Intron 17</td>
<td>VNTR</td>
<td>&lt;1683</td>
<td>5'-ATGGAGGATCATACCCCTGTATG-3'</td>
</tr>
<tr>
<td>125570</td>
<td>Intron 17</td>
<td>[54 nt]ₙ, repeat</td>
<td>&lt;636</td>
<td>5'-ACCTAACCTATTGGACCCAGTCTCCC-3'</td>
</tr>
<tr>
<td>156504</td>
<td>Intron 20</td>
<td>VNTR</td>
<td>&lt;636</td>
<td>5'-CCTGTAATGCTCCCTATAAT-3'</td>
</tr>
<tr>
<td>157139</td>
<td>Intron 17</td>
<td>[CTTT(T)]ₙ₀, repeat</td>
<td>5'-AATTACAGGTTGTTGG-3'</td>
<td></td>
</tr>
</tbody>
</table>

* Position of the 5’ end of the primer in the genomic sequence of Rb gene with respect to the translation start codon.
PCR-SSCP and Sequencing Analysis of p53 Mutations. PCR-SSCP was performed using primer sets and PCR conditions that we described previously (38, 39), and a complete description of the procedures of this analysis is provided in the primary report on the p53 mutational analysis (39). To avoid possible PCR errors introduced during the genomic DNA amplification, shift-band retrieval after SSCP, and cyclic sequencing, we performed sequencing from both directions, and the sequencing result from every case was confirmed by an independent repeat analysis.

pRb and p53 Immunohistochemistry. Immunohistochemistry of paraffin sections of the tumor and normal specimen was performed as described previously (40, 41). In brief, after dewaxing and rehydration, each 5-μm section was first treated with Target Unmasking Fluid (PharMingen, San Diego, CA) in a 800-W microwave oven at 10% power for 10 min, then with 1% H2O2 to quench the endogenous peroxidase activity. After blocking the nonspecific protein binding with 2% normal serum, sections were incubated with 2 μg/ml primary monoclonal pRb antibody PMG3–245 (PharMingen) or p53 antibody Ab-6 (OncoGene Science, Cambridge, MA) at room temperature for 2.5 h, then with a secondary biotinylated anti-primary antibody for 1 h, and finally with avidin-biotin-peroxidase complex (Vector ABC Elite Kit; Vector Laboratories, Burlingame, CA) for 45 min. Staining was visualized using Peroxidase Substrate Kit (3,3′-diaminobenzidine; Vector Laboratories). Hematoxylin was used as the nuclear counterstain in pRb staining. To ensure reproducibility, three consecutive slides were stained in separate experiments. The neighboring esophageal epithelia were always used as a positive control to qualify the positive staining in tumors for every case.

Because the pRb staining intensity in the cancer cells was comparable to that of the normal cells, based on estimation of percentage of the positively stained cells in the cancer nests, pRb immunostaining for cancer was graded as follows: (a) +++, >40% of the cells positively stained; (b) +, 10–40% of cells positively stained; (c) +, <10% of cells positively stained; and (d) −, no cells positively stained. For the p53 immunostaining, level of immunoreactivity was graded based on the intensity of the staining as well as the distribution of positively stained cells in the tumor. The four resulting classes were: (a) +++, very strong p53 staining in almost all of the cancerous cells over the entire tumor region; (b) +++, p53 staining with moderate intensity or only cells of the peripheral regions of 10–40% of the cancer nests were intensively stained; (c) +, weak p53 staining or only cells of the peripheral region of <10% of the cancer nests showed moderate to intensive p53 staining; and (d) −, no detectable p53 staining in cancerous cells.

Statistical Methods. The χ2 test with four degrees of freedom was used to assess the relationship between LOH of the Rb gene and the immunohistochemical staining pattern of pRb protein as well as the relationship between p53 alteration and pRb staining. The odds ratio was calculated in a 2 × 2 table, with the first column corresponding to the sample group designated as baseline level and the second being the sample group to be compared. In case of the 2 × 5 table, the group in column 1 was always designated as baseline in the analysis of each of the sample group in the subsequent columns. Fisher’s exact test was used to evaluate correlation in the 2 × 2 tables.

RESULTS
LOH Analysis of Rb Gene Using RFLP and VNTR Markers. The Rb gene spans ~200 kb on chromosome 13q21. To determine its allelic status over its entire length, we used four intragenic polymorphic sequences as genetic markers. These markers reside at 2, 99, 124, and 157 kb, respectively, downstream of the translation initiation codon of the Rb gene and, therefore, specifically reveal the allelic status of the Rb gene itself. As shown in Tables 2 and 3, of the 56 cases of ESCC we analyzed, 55 were informative for at least one of the four polymorphic markers. LOH was detected in 9 of 25 of the tumors informative for intron 1 RFLP marker, in 19 of 38 of the tumors informative for intron 17 RFLP, in 16 of 34 of the tumors informative for intron 17 VNTR marker, and in 8 of 26 of the tumors informative for intron 20 VNTR (Fig. 1). In summary, 30 of 55 (55%) tumors exhibited LOH in at least one of the four markers being analyzed (designated as Rb+/−), 26 tumors did not have detectable LOH at the Rb locus (Rb+/-). Notably, the frequencies of LOH over the entire region of the Rb gene were not uniform. LOH was more frequently observed in the central region (intron 17) than in the proximal and distal regions (introns 1 and 20). Furthermore, many LOH events had a detectable regional confinement and may not always affect an entire allele of the Rb gene. For example, among the four Rb+− samples that were informative for all four polymorphic loci, three showed LOH only in the distal loci but

Table 2 Summary of LOH results of four polymorphic markers in the Rb locus

<table>
<thead>
<tr>
<th>Marker</th>
<th>Intron 1</th>
<th>Intron 17</th>
<th>Intron 17</th>
<th>Intron 20</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOH</td>
<td>9 (36%)</td>
<td>19 (50%)</td>
<td>16 (53%)</td>
<td>8 (31%)</td>
<td>30 (55%)</td>
</tr>
<tr>
<td>No-LOH</td>
<td>14</td>
<td>19</td>
<td>14</td>
<td>18</td>
<td>55</td>
</tr>
<tr>
<td>ND</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* a Informative for at least one marker.
  b Contain LOH in at least one marker.
  c No LOH detected at either of the informative markers.
  d ND, not determined. These samples are not included in the estimation of LOH percentage.
  e Allelic status not determined in either of the informative marker.
Table 3  Alterations of the Rb and p53 genes in human ESCC

<table>
<thead>
<tr>
<th>No pRb-positive cells</th>
<th>Sparse pRb-positive staining</th>
<th>Intermediate pRb staining</th>
<th>Extensive pRb staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rb</strong></td>
<td><strong>pRb</strong></td>
<td><strong>p53</strong></td>
<td><strong>Rb</strong></td>
</tr>
<tr>
<td>No.</td>
<td>LOH</td>
<td>IHC</td>
<td>mutation</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>-</td>
<td>del 2</td>
</tr>
<tr>
<td>22</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>34</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>35</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>52</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>57</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>68</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>50</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>58</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>67</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>70</td>
<td>+</td>
<td>-</td>
<td>++(i4)</td>
</tr>
<tr>
<td>61</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>38</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>62</td>
<td>N1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Samples were grouped according to distinctive pRb patterns from immunohistochemistry. IHC, immunohistochemistry; ND, not determined; NI, noninformative.

b 1, has LOH; - , no LOH.

- , + , and +++ , increasing levels of immunostaining; - , no staining; s , scattered staining; f , focal staining.

c - , no mutation; + , missense mutation; st, nonsense mutation; ix, mutation in intron x; del x, microdeletion of x nucleotides; int x, insertion of x nucleotides.

d - , no alteration (LOH and/or IHC = +++ for Rb, mutation and/or IHC = +++ for p53); - , no alteration (LOH and/or IHC = +++ for Rb, mutation and/or IHC = +++ for p53); h, heterogeneous cancer nest.
not in the proximal loci, and one had the opposite orientation. Of 30 tumor samples containing LOH, 13 were found to contain LOH in marker(s) at one end of the gene while both alleles of the marker(s) were retained at the other end (data not shown). The remaining 17 samples exhibited LOH in all of their informative markers.

Abnormality of pRb Expression in Primary ESCC. In all of the morphologically normal esophageal squamous epithelia adjacent to the tumors, extensive positive pRb immunostaining was confined in the nuclei, and all positively stained cells were in the parabasal layers of the epithelia (Fig. 2a). For the primary tumors, positive pRb staining was observed in 45 of the 56 (80%) cases. The intensity and subcellular location of the staining in the tumor was similar to that observed in the normal epithelia. However, the numbers of the pRb-positive cells in the tumor varied significantly among different tumor samples. Sixteen cases displayed extensive pRb staining (+ + + + ), 14 cases had an intermediate extent of staining (++), 15 cases only showed sparse positive staining (+) and 11 cases contained no pRb-positive cells (Table 3). Fifty-three of the 56 cases were well-differentiated SCC, characterized by keratinized cells and keratin-pearl in the internal layers of cancer nests. pRb-positive cells were observed in the peripheral layers of the cancer nests. The remaining three cases of the ESCC were poorly differentiated and had no keratinized cells in the cancer. pRb staining was sparse (+) in two of these three cases, and the positively stained cells were scattered in the cancer nests; the other case contained no pRb staining.

In the 16 cases of extensively stained (+ + + + ) tumors, at least 40% of the cells in every cancer nests were pRb positive, all of which are in the peripheral layers (Fig. 2b). This type of staining was similar to that of the adjacent normal tissues. Of the 14 cases with intermediate (+ ++ ), 7 exhibited a focal pattern, in which positive staining were observed in ~60% of the cancer nests and had an extent similar to that of the intensive (+ + + + ) staining; whereas in the remaining cancer nests, either no staining or very few stained cells could be detected (Fig. 2c).

Another 7 cases of intermediate pRb staining (+ + ) tumors showed a scattered staining pattern, in which all cancer nests had a low percentage (10–25%) of pRb-positive staining cells. Compared to the adjacent normal epithelia, the scattered intermediate (+ + ) staining as well as the sparse staining (+) and lack of staining (−) clearly demonstrate a significant decrease or suppression of the pRb expression in the entire cancer.

Correlation between LOH of the Rb Gene and Abnormal Expression of pRb Protein. Status of pRb protein expression was compared with the allelic status of the Rb gene (Table 4). In cases with extensive staining (+ + + + ) of pRb protein, LOH of the Rb gene was detected in only 1 of 16 cases. In the 7 cases with heterogeneous cancer nests in term of pRb expression, only 2 cases had Rb LOH. Whereas in cases in which pRb expression was only seen in a small fraction of tumor cells (scattered ++ or ++ ), Rb LOH was observed in 5 of 7 and 12 of 14 informative cases, respectively. In the 11 informative cases with no detectable pRb expression, 10 contained LOH in the Rb gene. Compared to tumors with extensive pRb expression, there is an high odds ratio for the presence of Rb LOH in tumors with no or low extensive pRb expression. The association of Rb LOH with this abnormality of pRb expression is statistically significant (P < 0.0001; odds ratio: 4.2).

Relationship between Rb and p53 Alterations. Status of the p53 gene in the ESCC was examined by immunohistochemistry and mutation analysis (39). The p53 immunostaining pattern was classified as ++ + + , ++ + , or − (Fig. 3), and the p53 immunopositivity was correlated with gene mutation. Therefore, in this study, p53 alteration was defined by either the presence of mutation(s) in the gene sequence, p53 accumulation, or − (Fig. 3), and the p53 immunopositivity was correlated with gene mutation. Therefore, in this study, p53 alteration was defined by either the presence of mutation(s) in the gene sequence, p53 accumulation, or − (Fig. 3), and the p53 immunopositivity was correlated with gene mutation. Therefore, in this study, p53 alteration was defined by either the presence of mutation(s) in the gene sequence, p53 accumulation, or − (Fig. 3), and the p53 immunopositivity was correlated with gene mutation. Therefore, in this study, p53 alteration was defined by either the presence of mutation(s) in the gene sequence, p53 accumulation, or − (Fig. 3), and the p53 immunopositivity was correlated with gene mutation. Therefore, in this study, p53 alteration was defined by either the presence of mutation(s) in the gene sequence, p53 accumulation, or − (Fig. 3), and the p53 immunopositivity was correlated with gene mutation. Therefore, in this study, p53 alteration was defined by either the presence of mutation(s) in the gene sequence, p53 accumulation, or − (Fig. 3), and the p53 immunopositivity was correlated with gene mutation.
extensive pRb staining (+++) in all cancer nests together with no Rb LOH (Rb+/+) are considered to be indicative of a normal Rb. In the five Rb+/+ tumors in which pRb staining had a focal ++ pattern, Rb status were not defined because of their heterogeneous composition of both pRb-extensive and pRb-negative cancer nests. This criterion, however, did not significantly change the number of ESCC cases with Rb alterations as determined by Rb LOH along (only 6 more cases were recognized as having Rb alteration). Altogether, Rb alteration was observed in 36 (71%) of the 51 cases with defined Rb status (Table 3).

---

**Table 4** Relationship of pRb immunostaining with Rb LOH and p53 alterations

<table>
<thead>
<tr>
<th>pRb immunoreactivity</th>
<th>+ +</th>
<th>Focal</th>
<th>Scattered</th>
<th>+</th>
<th>-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb+/+</td>
<td>15</td>
<td>2</td>
<td>5 (71%)</td>
<td>12 (86%)</td>
<td>10 (92%)</td>
<td>30 (56%)</td>
</tr>
<tr>
<td>Rb+/−</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Rb−/−</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>p53 alteration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>7</td>
<td>4 (57%)</td>
<td>6 (86%)</td>
<td>12 (80%)</td>
<td>11 (100%)</td>
<td>40 (71%)</td>
</tr>
<tr>
<td>−</td>
<td>9</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>

a NI, not informative; OR, odds ratio.

b OR of pRb staining of each level with Rb LOH. Overall correlation was performed by $\chi^2$ test (degree of freedom = 4); $P < 0.0001$.

c OR of pRb staining of each level with p53 alteration. Overall correlation was performed by $\chi^2$ (degree of freedom = 4); $P = 0.015$.

---

Fig. 2 Different extents of pRb immunostaining in human ESCC. a, pRb staining (brown) in the normal esophageal epithelium; b, +++, positively stained cells were in the peripheral layers of the cancer nest; c, focal +++, the cancer nest at the bottom left showed extensive staining, whereas the top right nest contained only sparse pRb-positive cells; d, +, few cells in the peripheral layers of the cancer nest showed positive staining.

---

Downloaded from clincancerres.aacrjournals.org on April 19, 2017. © 1999 American Association for Cancer Research.
Fig. 3 Different levels of p53 immunostaining in human ESCC. 

- **a**, strong p53 immunoreactivity (+++);
- **b**, intermediate immunoreactivity (++);
- **c**, weak immunoreactivity (+).
these 51 ESCC cases, alteration of the Rb gene was strongly associated with the alteration of the p53 gene (P = 0.0015; Table 5). Specifically, 31 of the tumors contained alterations in both Rb and p53 genes, whereas only 6 or 5 samples contained alteration of only p53 or Rb, respectively. Nine cases did not contain detectable alteration in either of the genes.

**DISCUSSION**

In this study, we analyzed the allelic status of the Rb gene in ESCC specimens from a high-risk population in northern China. Consistent with previous observations in ESCC (16, 17), we found that 30 (55%) of the 55 informative cases had LOH of the Rb gene. Allelic imbalance of the Rb gene in these tumors was associated with significant decrease in pRb protein expression. Among the 56 cases we studied, substantial alteration of pRb expression, ranging from low extent (scattered + + or +) to no detectable expression (−), occurred in 37 cases. Remarkably, 27 (90%) of the 30 Rb+/− tumors showed this altered pRb expression, whereas only 5 (20%) of the 25 Rb+/+ tumors showed altered pRb expression. The association between Rb LOH and altered pRb expression was very significant (P < 0.0001; Table 2).

Frequent LOH at specific locus compared to the infrequent (∼10%) random LOH at loci with no obvious etiologic roles usually implies the involvement of tumor suppressor gene(s) that affected region in the pathogenesis of cancer (16, 42). Association between LOH on 13q where Rb is located and decreased pRb expression was observed in many tumors, such as bladder, liver, and lung cancers (9, 10, 18). In other cancers such as head and neck SCC, however, there was a lack of concordance between LOH on 13q and pRb protein staining, and this led to the suspicion that other potential tumor suppressor gene(s) reside near this region (13). Although it is possible that Rb inactivation may play different roles in the formation of different cancers, the lack of concordance between pRb expression and the “apparent” Rb LOH in some cases could have also resulted from the particular markers used in the LOH analysis. Polymorphic loci that are remote from the gene of interest may not accurately reveal the LOH rate of the target gene (43, 44). Unfortunately, we did not observe the LOH at Rb locus had obvious regional confinement in at least some of the ESCC cases we analyzed. In this study, all markers that we chose reside directly within the gene, and therefore, the high LOH rate we observed in our ESCC samples is believed to reveal the actual allelic status of their Rb gene. Furthermore, we observed higher LOH frequency (50–53%) in the middle and lower frequency (31–36%) in the proximal and distal ends of the Rb gene, suggesting LOH is centered at the Rb gene rather than at some other loci beyond the gene. The strong association between Rb LOH and decreased pRb expression suggests that Rb LOH is a good indicator of the inactivation of the Rb gene in ESCC.

As the pRb staining decreased in groups with ++ +, ++ +, +, and −, there was a trend of increase in the frequency of Rb LOH (Table 4). This relationship may be due to the loss of the normal Rb allele during different stages of tumorigenesis. An early loss may lead to complete loss of pRb expression in all cancer cells, whereas such an event at a later stage may leave some cancer cells remaining genotypically heterozygous. Due to the growth advantage of the Rb+/− cells, they usually significantly outnumber the Rb+/+ cells in the cancer and, therefore, cause the cancer to appear Rb+/−/IHC+/++. However, a significant number of Rb+/+ cells sometimes may still be capable of masking the allelic status of the majority cancer cells in a PCR-based LOH analysis and making the cancer appear Rb+/−/IHC+/

<table>
<thead>
<tr>
<th>Rb alteration</th>
<th>p53 alteration</th>
<th>odds ratio</th>
<th>CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>31</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>6</td>
<td>31</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5 Correlation between Rb and p53 alterations

* Odds ratio = 9.3 (SE, 6.6). Fisher’s exact test, P = 0.0015.
that alterations in \( Rb \) and \( p53 \) functions have a cooperative effect on the progression in bladder and non-small cell lung carcinoma and on lowering the survival rate of the patients (19, 20, 21). It has been postulated that aberrant \( Rb \) and \( p53 \) can deregulate cell cycle control and reduce the ability of the abnormal cells to undergo apoptosis (37). The imbalance produced by an enhanced proliferative activity and reduced apoptotic rate may greatly increase growth advantage of the affected cells, accelerating the carcinogenic process and increasing invasiveness. The strong association between \( Rb \) and \( p53 \) alteration in our case suggests that such dual alterations may cooperatively produce a tumorigenic effect during ESCC formation. To test this hypothesis, however, it is necessary to obtain evidence of \( Rb \) and \( p53 \) alteration in the early-stage ESCC, for example, from studying biopsy samples.

ESCC development is a multistage process involving multiple genetic changes (47). The frequent and strongly associated \( Rb \) and \( p53 \) alterations that we observed led us to propose that concomitant \( Rb \) and \( p53 \) inactivation may be the major event involved in the pathogenesis and progression of ESCC due to the superior selective advantage of the affected cells. In addition, alterations of other genes in the \( Rb \) pathway (e.g., \( p16/p15 \) and \( cyclin \ D1 \)) and \( p53 \) pathway (e.g., \( Bax \) and \( p21 \)) may also be involved in promoting malignant transformation by accelerating proliferation and suppressing apoptosis synergistically. Indeed, we have analyzed 12 of the 15 \( Rb \) normal cases shown in Table 3 and found that 9 cases contained alterations in \( p16, p15, \) or both (48). Therefore, almost all ESCC cases we analyzed were \( Rb \) and \( p53 \) alteration in the early-stage ESCC, for example, from studying biopsy samples.

**ACKNOWLEDGMENTS**

We thank Jingshang Zhang of the Department of Statistics at Rutgers University for his suggestions in the statistical analysis of our data and for his help in performing and verifying the relevant calculations.

**REFERENCES**


Human Esophageal Cancer

Alteration in \( p53 \) Protein Expression and Associates with \( p53 \) Alteration in Human Esophageal Cancer

Eric Poe Xing, Guang-Yu Yang, Li-Dong Wang, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/5/5/1231

Cited articles
This article cites 46 articles, 18 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/5/5/1231.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/5/5/1231.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.