Loss of Heterozygosity of the \textit{Rb} Gene Correlates with pRb Protein Expression and Associates with \textit{p53} Alteration in Human Esophageal Cancer\textsuperscript{1}

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ABSTRACT

To understand the alterations of \textit{Rb} tumor suppressor gene and the relationship between defects in the \textit{Rb} and \textit{p53} pathways in human esophageal carcinoma, we examined the loss of heterozygosity (LOH) of the \textit{Rb} gene and immunohistochemical staining of pRb protein in 56 esophageal squamous cell carcinoma specimens and related the results to the \textit{p53} gene alterations. Using four intronic polymorphic markers as probes, we observed LOH of the \textit{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 \textit{Rb} status. In the tumor samples containing \textit{Rb} LOH, 90\% showed low or no pRb expression, whereas in samples without \textit{Rb} LOH, only 20\% had altered pRb expression. There was a strong association between LOH of the \textit{Rb} gene and alteration of pRb expression in our samples \textit{(P < 0.0001)}, suggesting LOH is a main event leading to \textit{Rb} inactivation. We found that \textit{Rb} LOH was more frequent in tumors with \textit{p53} mutations \textit{(P < 0.05)}, which occurred in 31 of the 49 cases analyzed. When the status of \textit{Rb} and \textit{p53} alterations was evaluated by the combined results of immunohistochemical and genetic analyses, we found that alteration of \textit{Rb} and \textit{p53} had an even stronger association in our esophageal squamous cell carcinoma samples \textit{(P = 0.0015)}.

Among the 51 cases in which both the \textit{Rb} and \textit{p53} status were determined, 31 contained alterations in both genes, and only 5 and 6 cases were altered in only \textit{Rb} and only \textit{p53}, respectively. Our results suggest that defects in the \textit{Rb} and \textit{p53} pathways and their potential synergistic effect in deregulating cell cycle and apoptosis are major mechanisms for esophageal carcinogenesis.

INTRODUCTION

The \textit{Rb} gene, located on chromosome 13\textit{q}14.2, was the first tumor suppressor gene to be identified in humans and was initially determined to be associated with the development of retinoblastoma \textit{(1)}. \textit{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 \textit{Rb} pathway \textit{(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 \textit{G}_{1} \textit{(3)}. Suppression of pRb function through hyperphosphorylation causes the release of the E2F factors and triggers a burst of gene expressions that facilitates \textit{G}_{1}-\textit{S} transition \textit{(4, 5)}. Functional loss of the \textit{Rb} gene frequently occurs in the carcinogenic processes of many types of cancer \textit{(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 \textit{(1, 8)}. Therefore, LOH\textsuperscript{4} at the \textit{Rb} locus is an important event reflecting potential functional alteration in the \textit{Rb} gene. LOH on 13\textit{q}, where \textit{Rb} is located, is a common feature in many types of cancer involving bladder, lung, breast, head and neck, and other organs \textit{(9–25)}. However, it is not always possible to observe concordance of LOH at 13\textit{q} and loss of pRb protein expression in different types of tumor, suggesting that either the selected LOH markers are for other genes near the \textit{Rb} locus or other mechanisms are involved in the inactivation of the \textit{Rb} gene. In human esophageal cancer, LOH at the \textit{Rb} locus was observed in 54\% of SCCs and in 36\% of adenocarcinomas \textit{(16, 17)}, but whether it leads to a decrease in pRb expression is not clear.

Recently, an association between aberrant pRb and \textit{p53} expression was observed in bladder and several other types of cancer \textit{(19–25)}\textsuperscript{1}. \textit{p53} is critical for coordinating multiple growth control checkpoints in response to genotoxic insults and abnormal proliferation \textit{(26)}. Wild-type \textit{p53} can block malignant cell

\textsuperscript{1}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.

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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 antia apoptotic 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 anti tumorigenic 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 intron 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 esophagei 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.

**Oligonucleotides for determining Rb LOH**

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*Position of the 5’ end of the primer in the genomic sequence of Rb gene with respect to the translation start codon.

**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 MgCl<sub>2</sub>, standard PCR buffer, and 1 unit of Taq polymerase (all from Life Technologies, Inc., Gaithersburg, MD). 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 (T<sub>a</sub>), and 2 min at 72°C for initial cycle; and then 30 cycles of 90 s at 95°C, 45 s at T<sub>a</sub>, and 45 s at 72°C, followed by 2 min at 72°C. T<sub>a</sub> cycle number, and Mg<sup>2+</sup> 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 (BamHI for intron 1 RFLP and XbaI 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<sup>33</sup>P]dATP, and PCR products were resolved on 6% denaturing polyacrylamide gel and radiographed for 4 h before analyzing the signals. Semi-quantitative 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.
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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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.

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 introgenic 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. 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 3  Alterations of the Rb and p53 genes in human ESCC

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Notes:
- Samples were grouped according to distinctive pRb patterns from immunohistochemistry. IHC, immunohistochemistry; ND, not determined; NI, noninformative.
- +, has LOH; −, no LOH.
- +, +++, and ++++, increasing levels of immunostaining; −, no staining; s, scattered staining; f, focal staining.
- %, no mutation; +, missense mutation; st, nonsense mutation; ix, mutation in intron x; del x, microdeletion of x nucleotides; int x, insertion of x nucleotides.
- +, has alteration (LOH + or IHC −+− for Rb, mutation + or IHC −+− for p53); −, no alteration LOH /IHC −+− for Rb, mutation /IHC −+− for p53; h, heterogeneous cancer nest.
staining was similar to that of the adjacent normal tissues. Of the 14 cases with intermediate (+ + +) staining, 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 mutation was defined by either the presence of mutation(s) in the gene sequence, p53 accumulation, 5 of which had intron (2 cases), frameshift (2 cases), or nonsense mutation (1 case). Full details of these mutations are provided in a separate report which addresses the distributions, spectrum, progression, and pathological relevance of these events (36). Altogether, 40 of 56 (71%) ESCC cases demonstrated alterations of the p53 gene. Compared to samples with + + + pRb staining, there is a moderate increase of the frequency of p53 alteration in ESCC samples with focal + + pRb staining (from 44 to 57%). A dramatic increase of the p53 alteration rate was seen in ESCC samples with scattered + + +, + +, and − pRb staining (86, 80, and 100%, respectively). Comparison of the Rb LOH with p53 mutation revealed that LOH of the Rb gene was significantly more frequent in tumors with p53 mutations than in tumors not detected to have p53 mutation (P = 0.017).

In this study, Rb alteration was defined by either a Rb+/− allelic type, low extent of pRb staining (scattered + +, +, and −) in all cancer nests, or both. Only a homogenous display of

Fig. 1. LOH at four polymorphic loci within the Rb gene in ESCC samples. Lanes N, normal; Lanes T, tumor. Matching pairs of normal and tumor DNA were located immediately adjacent to each other. a, amplification of the BamHI RFLP in intron 1 produced a segment of 195 bp. Restriction digestion resulted in fragments of 140 and 45 bp. LOH was recognized as a partial or complete loss of either the uncleaved (195 bp) or the cleaved (140 + 45 bp) allele. The first pair did not show LOH, the other two pairs all contained LOH at this loci. Residue signal left in the deleted allele probably resulted from the contaminating normal cell but not in the matching normal samples. b, PCR of XbaI RFLP in intron 17 generated a 190-bp band that can be digested into 115- and 75-bp fragments. Case 3 did not contain LOH at this locus, and the remaining cases were representative LOH cases. c, PCR of the [54 nt] VNTR in intron 17 gave a spectrum of alleles ranging in size from 1300 to ~1700 bp. Allelic imbalance was seen in the tumor samples of cases 9, 14, and 34 but not in the matching normal samples. d, intron 20 microsatellite [CTTT(T)], VNTR was amplified using PCR with radiolabeled primers and resolved on 6% polyacrylamide gel. Allelic imbalance was seen in tumors 24 and 22.

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 staining 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
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 alone (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).

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**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>1 (6%)</td>
<td>2 (14%)</td>
<td>5 (71%)</td>
<td>12 (86%)</td>
<td>10 (92%)</td>
<td>30 (56%)</td>
</tr>
<tr>
<td>Rb(^{+-/})</td>
<td>15</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>p53 alteration</td>
<td>2.14</td>
<td>10.73</td>
<td>19.20</td>
<td>19.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OR(^b)</td>
<td>6.0</td>
<td>37.5</td>
<td>90.0</td>
<td>150.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x^2) (Pearson’s)</td>
<td>0.35</td>
<td>3.49</td>
<td>4.29</td>
<td>9.28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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\(^a\) NI, not informative; OR, odds ratio.
\(^b\) OR of pRb staining of each level with Rb LOH. Overall correlation was performed by \(x^2\) test (degree of freedom = 4); \(P < 0.0001\).
\(^c\) OR of pRb staining of each level with p53 alteration. Overall correlation was performed by \(x^2\) (degree of freedom = 4); \(P = 0.015\).
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 etiological roles usually implies the involvement of tumor suppressor gene(s) of 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. Polyomorphic loci that are remote from the gene of interest may not accurately reveal the LOH rate of the target gene (43, 44). Association with Rb LOH has 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+/−/pRb+/−. 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+/−/pRb+/−. This may explain the observation that a few tumor samples with low extent pRb stain still showed Rb+/+ allelic type. Interestingly, we observed 7 cases of well-differentiated SCC displaying a phenotype between abnormal and normal pRb expression. These tumors had a focal + pRb staining in which ~20–40% of the cancer nests showed extensive pRb staining, whereas all remaining nests had no pRb staining. Allelic analysis showed that only 2 of the 7 such tumors contained Rb LOH; others had no detectable allelic imbalance. It is not known whether this heterogeneous phenotype of the cancer nests is due to a polyclonal origin of the cancer, to Rb loss in some of the cancer cells before formation of cancer nests, or to some unknown mechanism that down-regulates pRb expression in the pRb− nests. More functional studies are needed to determine the actual Rb status in these five Rb+/− tumors with focal + pRb staining.

In each of the three groups of ESCC showing altered pRb expression (scattered + +, +, and −), there were small numbers of cases (1 of 11, 2 of 14, and 2 of 7, respectively) containing no detectable Rb LOH at the four intronig loci. Because we did not examine other types of genetic alterations that had been reported in the Rb gene, such as homozygous deletion (45), aberrant methylation (46), or possible dominant negative mutations, it is highly possible that these mechanisms also, to some extent, contributed to the Rb inactivation. Also, we cannot exclude the possibility that some special types of LOH such as small deletion or point mutations on one allele remained undetected.

To understand the roles and the relationship between inactivation of the Rb and p53 pathways in human ESCC, we analyzed the correlation between alterations of Rb and p53 genes in the 51 tumor samples in which the status can be determined by a combination of immunohistochemical and genetic analyses. Remarkably, Rb alteration had a strong association with p53 alteration in these samples (P = 0.0015). In particular, in all of the 11 tumors with pRb− phenotype, which we believe to indicate most severe Rb inactivations, p53 alterations were ubiquitously present, and most of the alterations were identified by a mutation accompanied by +++ protein staining (Table 3).

Concomitant Rb and p53 abnormalities have been observed in a variety of cancers (19–25). Recent studies have suggested

| Table 5  Correlation between Rb and p53 alterations* |
|------------------|----------|----------|
| Rb alteration    | p53 alteration | − | + |
| +                | 6        | 31       |
| −                | 9        | 5        |

* 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 defective in the Rb pathway. It would be interesting to examine the tumors with no p53 alterations to see whether they contain defects in other members of the p53 pathway, such as the recently discovered putative p53 homologues p51 (49), p62 (50), and p73, or factors upstream (e.g., ATM) or downstream (e.g., Bax or p21WA1) of the p53 protein.

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REFERENCES


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, et al.