Loss of Heterozygosity of the *Rb* Gene Correlates with pRb Protein Expression and Associates with *p53* Alteration in Human Esophageal Cancer[1]

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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 introgenic 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 ($P = 0.0015$). Among the 51 cases in which both the *Rb* and *p53* status were determined, 31 contained alterations in both genes, and only 5 and 6 cases were altered in only *Rb* and only *p53*, respectively. Our results suggest that defects in the *Rb* and *p53* pathways and their potential synergistic effect in deregulating cell cycle and apoptosis are major mechanisms for esophageal carcinogenesis.

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 G_1 (3). Suppression of pRb function through hyperphosphorylation causes the release of the E2F factors and triggers a burst of gene expressions that facilitates G_1-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, LOH[4] 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

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[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.

Table 1 Oligonucleotides for determining *Rb* LOH

Primer no.	Locus	Polymorphism type	Product size (bp)	Primer sequence
2162[a]	Intron 1	RFLP	195	5′-CAGGACAGCGGCCCGGAG-3′
2356		*Bam*HI		5′-CTGCAGACGCTCCGCCGT-3′
99311	Intron 17	RFLP	190	5′-TCCCACCTCAGCCTCCTTAG-3′
99500		*Xba*I		5′-GTAGGCCAAGAGTGGCAGCT-3′
123888	Intron 17	VNTR	<1683	5′-ATGAGGGATCCACCCCCCTGATGT-3′
125570		$[54\ nt]_{30}$ repeat		5′-ACCTAAGCTATGGACCAAGTTTCC-3′
156504	Intron 20	VNTR	<636	5′-CTTGTAATATGCCTCATAAT-3′
157139		$[CTTT(T)]_{20}$ repeat		5′-AATTAACAAGGTGTGGTGG-3′

[a] Position of the 5′ end of the primer in the genomic sequence of *Rb* gene with respect to the translation start codon.

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 introgenic 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 $MgCl_2$, 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 (T_a), and 2 min at 72°C for initial cycle; and then 30 cycles of 90 s at 95°C, 45 s at T_a, and 45 s at 72°C, followed by 2 min at 72°C. T_a, cycle number, and Mg^{2+} 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 (*Bam*HI for intron 1 RFLP and *Xba*I 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-^{33}P]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 2 Summary of LOH results of four polymorphic markers in the *Rb* locus

	RFLP		VNTR		
	Intron 1	Intron 17	Intron 17	Intron 20	Total
Informative	25	38	34	26	55[a]
LOH	9 (36%)	19 (50%)	16 (53%)	8 (31%)	30 (55%)[b]
No LOH	14	19	14	18	25[c]
ND[d]	2	0	4	0	0[e]

[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.

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% H_2O_2 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 χ^2 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 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 (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 3 Alterations of the *Rb* and *p53* genes in human ESCC[a]

No pRb-positive cells							Sparse pRb-positive staining							Intermediate pRb staining							Extensive pRb staining						
No.	*Rb* LOH[b]	pRb IHC[c]	*p53* mutation[d]	*p53* IHC[c]	*Rb* status[e]	*p53* status[e]	No.	*Rb* LOH[b]	pRb IHC[c]	*p53* mutation[d]	*p53* IHC[c]	*Rb* status[e]	*p53* status[e]	No.	*Rb* LOH[b]	pRb IHC[c]	*p53* mutation[d]	*p53* IHC[c]	*Rb* status[e]	*p53* status[e]	No.	*Rb* LOH[b]	pRb IHC[c]	*p53* mutation[d]	*p53* IHC[c]	*Rb* status[e]	*p53* status[e]
9	+	−	del 2	−	+	+	5	+	+	+	+	+	+	1	+	++s	del 3	++	+	+	28	+	+++	+	++	+	+
22	+	−	+	+++	+	+	6	+	+	+	+	+	+	2	+	++s	−	−	+	−	3	−	+++	del 3	++	−	+
30	+	−	+	+++	+	+	7	+	+	−	−	+	−	4	+	++s	+	+++	+	+	16	−	+++	+(st)	−	−	+
34	+	−	+	+	+	+	13	+	+	+	+++	+	+	8	+	++s	int 5	++	+	+	18	−	+++	−	−	−	−
35	+	−	+	+++	+	+	14	+	+	ND	+	+	+	27	+	++s	ND	++	+	+	21	−	+++	−	−	−	−
52	+	−	+	+++	+	+	20	+	+	+	+	+	+	19	−	++s	+	+	+	+	25	−	+++	−	−	−	−
57	+	−	+	+++	+	+	24	+	+	+	++	+	+	51	−	++s	+	++	+	+	36	−	+++	+	+++	−	+
68	+	−	+	+++	+	+	26	+	+	−	+	+	+	31	+	++f	−	−	+	−	40	−	+++	−	−	−	−
50	+	−	ND	++	+	+	33	+	+	+	++	+	+	39	+	++f	+	+	+	+	43	−	+++	−	−	−	−
58	+	−	−	++	+	+	41	+	+	+	++	+	+	32	−	++f	+(i5)	−	h	+	44	−	+++	+	++	−	+
67	−	−	−	+	+	+	65	+	+	−	−	+	−	53	−	++f	−	−	h	−	46	−	+++	−	−	−	−
							70	+	+	+(i4)	−	+	+	59	−	++f	ND	−	h	−	47	−	+++	−	−	−	−
							61	−	+	ND	++	+	+	63	−	++f	+	+++	h	+	48	−	+++	del 2	−	−	+
							38	−	+	+	++	+	+	64	−	++f	ND	+	h	+	54	−	+++	−	+	−	+
							62	NI	+	−	−	+	−								55	−	+++	−	−	−	−
																					60	−	+++	−	−	−	−

[a] Samples were grouped according to distinctive pRb patterns from immunohistochemistry. IHC, immunohistochemistry; ND, not determined; NI, noninformative.
[b] +, has LOH; −, no LOH.
[c] +, ++, and +++, increasing levels of immunostaining; −, no staining; s, scattered staining; f, focal staining.
[d] −, no mutation; +, missense mutation; st, nonsense mutation; i*x*, mutation in intron *x;* del *x*, microdeletion of *x* nucleotides; int *x*, insertion of *x* nucleotides.
[e] +, has alteration (LOH^{+} and/or $ICH^{-,+,++s}$ for Rb, $mutation^{+}$ and/or $IHC^{+,++,+++}$ for p53); −, no alteration LOH^{-}/IHC^{+++} for Rb, $mutation^{-}/IHG^{-}$ for p53); h, heterogeneous cancer nest.

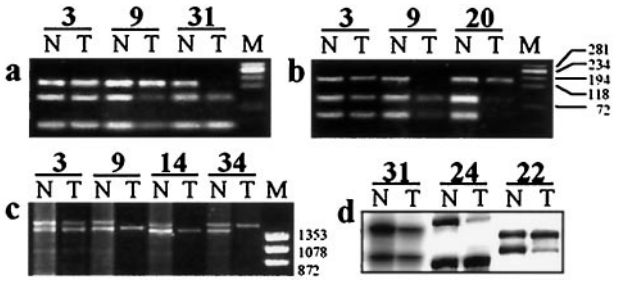

Fig. 1 LOH at four polymorphic loci within the *Rb* gene in ESCC samples. *Lanes N*, normal; *Lanes T*, tumor. Matching pairs of normal and tumor DNA were located immediately adjacent to each other. *a*, amplification of the *Bam*HI RFLP in intron 1 produced a segment of 195 bp. Restriction digestion 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 within the tumor tissue. *b*, PCR of *Xba*I 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 $[54\ nt]_n$ 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 $[CTTT(T)]_n$ VNTR was amplified using PCR with radiolabeled primers and resolved on 6% polyacrylamide gel. Allelic imbalance was seen in tumors *24* and *22*.

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. 2*a*). 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 nest. 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. 2*b*). This type of 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. 2*c*). 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 (+; Fig. 2*d*) 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 in the cancer cells, or both (Table 3). In the 56 ECSS cases, 35 had different levels of p53 protein accumulation, 26 of which had *p53* mutation (including 23 missense mutation, 1 frameshift insertion, and 2 in-frame deletions); 21 had no p53 protein 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

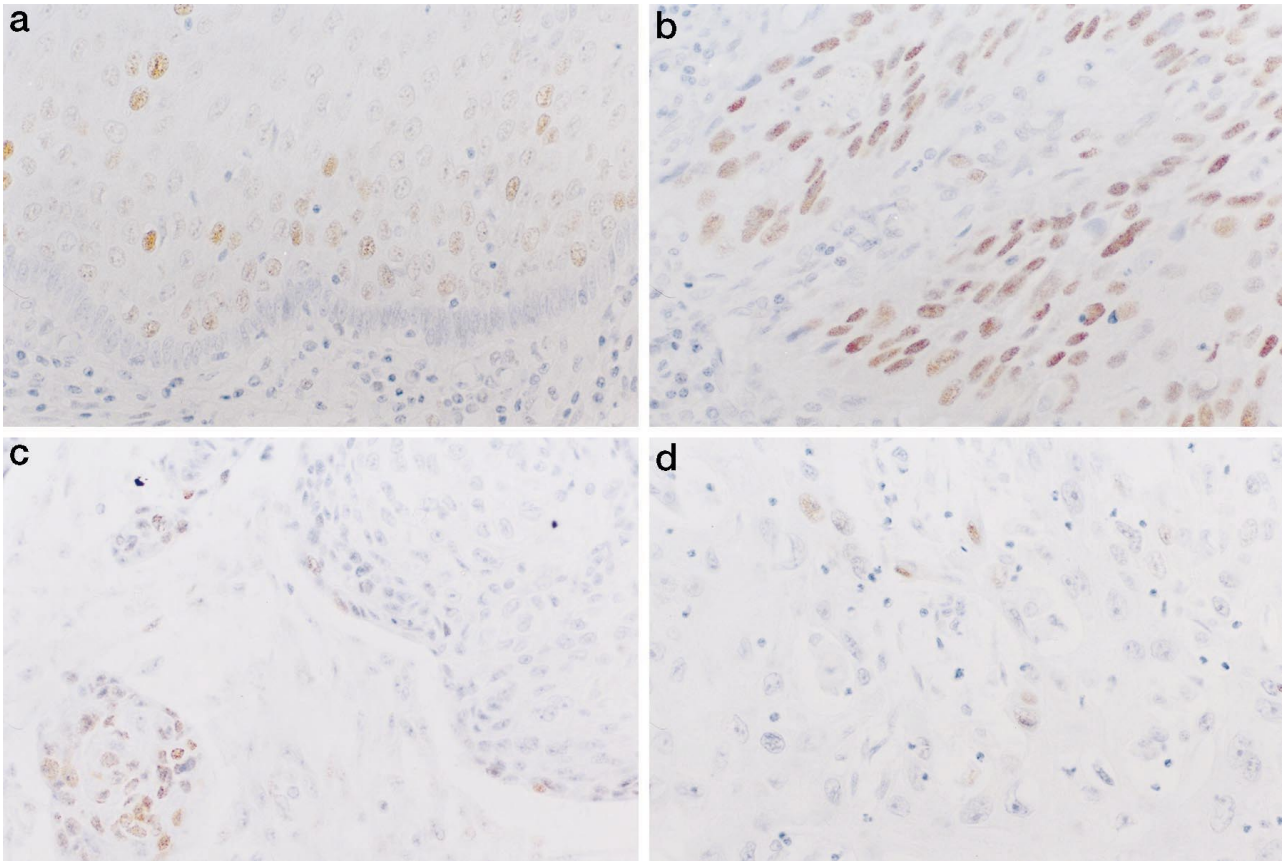

Fig. 2 Different extents of pRb immunostaining in human ESCC. *a*, pRb staining (*brown*) in the normal esophageal epithelium; *b*, +++, positively stained cells were in the peripheral layers of the cancer nest; *c*, focal ++, the cancer nest at the *bottom left* showed extensive staining, whereas the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

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

	pRb immunoreactivity					
		++				
	+++	Focal	Scattered	+	−	Total
$Rb^{+/-}$	1 (6%)	2 (14%)	5 (71%)	12 (86%)	10 (92%)	30 (56%)
$Rb^{+/+}$	15	5	2	2	1	25
NI[a]	0	0	0	1	0	1
χ^2 (Pearson's)		2.14	10.73	19.20	19.35	
OR[b]		6.0	37.5	90.0	150.0	
p53 alteration						
+	7 (44%)	4 (57%)	6 (86%)	12 (80%)	11 (100%)	40 (71%)
−	9	3	1	3	0	16
χ^2 (Pearson's)		0.35	3.49	4.29	9.28	
OR[c]		1.7	7.7	5.1	∞	

[a] NI, not informative; OR, odds ratio.
[b] OR of pRb staining of each level with *Rb* LOH. Overall correlation was performed by χ^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 χ^2 (degree of freedom = 4); $P = 0.015$.

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). In

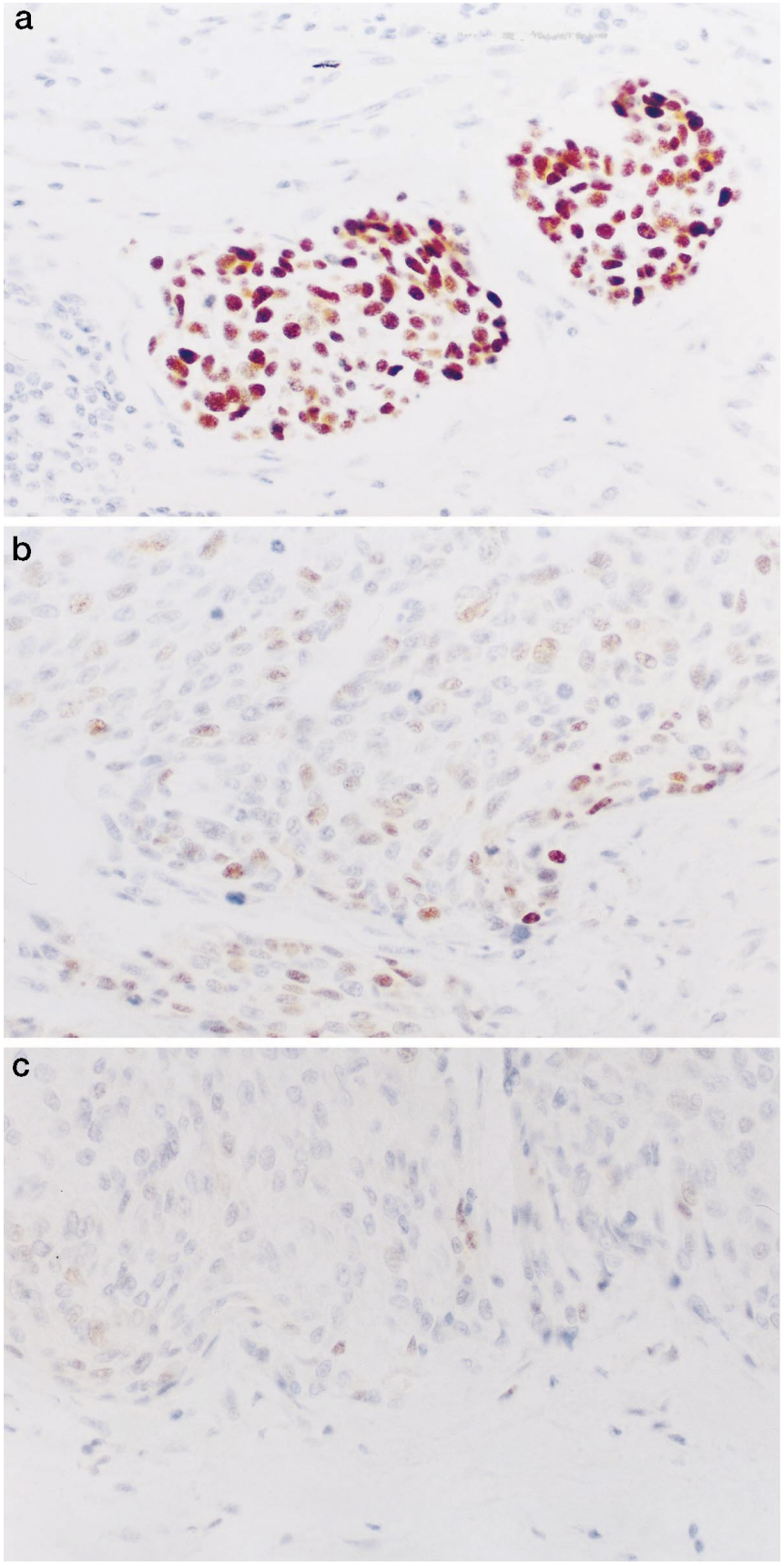

Fig. 3 Different levels of p53 immunostaining in human ESCC. *a*, strong p53 immunoreactivity (+++); *b*, intermediate immunoreactivity (++); *c*, weak immunoreactivity (+).

Table 5 Correlation between *Rb* and *p53* alterations[a]

	Rb alteration	
	−	+
p53 alteration		
+	6	31
−	9	5

[a] Odds ratio = 9.3 (SE, 6.6). Fisher's exact text, $P = 0.0015$.

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. Polymorphic loci that are remote from the gene of interest may not accurately reveal the LOH rate of the target gene (43, 44). Supporting this, we noticed that 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$^{+,++}$. 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 introgenic 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

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 $p21^{WAF1}$) of the p53 protein.

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