Advances in Brief

Frequent Loss of Heterozygosity at the Retinoblastoma Locus in Human Esophageal Cancers

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Abstract

Abnormalities in the retinoblastoma tumor suppressor gene (Rb) have been observed in a large number of human cancers. Loss of heterozygosity is a common mode of allelic inactivation of Rb and other tumor suppressor genes. We investigated DNA from 61 primary human esophageal tumors for loss of heterozygosity at the Rb locus using a polymerase chain reaction-based restriction fragment length polymorphism assay. Of informative cases, we found loss of heterozygosity in 14 of 26 (54%) squamous cell carcinomas and 5 of 14 (36%) adenocarcinomas. These data support the hypothesis that Rb inactivation is involved in the pathogenesis and/or progression of esophageal cancer.

Introduction

Lesions at various genetic loci have been reported in human esophageal cancer. These include amplification of a number of protooncogenes (1-4), LOH on chromosome 17p (4, 5), and point mutations within the p53 tumor suppressor gene (6). The retinoblastoma gene (Rb) has been implicated in the pathogenesis or progression of a broad range of human malignancies (7-16). A prototypic tumor suppressor gene, Rb is believed to contribute to carcinogenesis when both chromosomal copies of the gene are inactivated. Mechanisms of Rb inactivation include point mutation, rearrangement, and deletion, which can be intragenic or more extensive (7-16). There are two histological types of esophageal carcinoma: squamous cell carcinoma, and adenocarcinoma. Squamous cell carcinoma is associated with ethanol and tobacco use. Adenocarcinoma arises within Barrett's esophagus, a metaplastic columnar epithelium that develops as a complication of chronic gastroesophageal reflux (17). This study was undertaken to determine the frequency of LOH at the Rb gene locus, or Rb-LOH, in human primary esophageal carcinoma. We examined DNA from 41 esophageal squamous cell carcinomas and 20 adenocarcinomas for Rb-LOH using RFLP assays based on the PCR.

Materials and Methods

Tissue Preparation and DNA Extraction. Tissue samples were obtained at endoscopy or surgery from patients with esophageal cancer.

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The abbreviations used are: LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction.

In 40 of 41 squamous cell carcinomas and 10 of 20 adenocarcinomas, the specimens were frozen in liquid nitrogen immediately after removal. Homologous normal tissue was obtained from normal gastric or normal esophageal mucosa in all patients. At endoscopies, after informed consent was obtained, 6-8 biopsies were taken from normal stomach; approval was granted by the Human Volunteers Research Committee for this study. At surgeries, a large piece (1-2 cm³) of normal gastric mucosa was dissected from the resection specimen. Tumor type was verified by histological examination. Whenever possible, tumors were purified by cryostat sectioning and microdissection as described (5, 18).

One of 40 squamous cell carcinomas and 10 of 20 adenocarcinomas were subjected to flow cytometric cell sorting, by which aneuploid tumor nuclei can be separated from contaminating diploid stromal nuclei on the basis of DNA content. These samples were obtained from esophagectomy specimens at the time of surgery and frozen at -70°C until processed. Nuclei were isolated from tumor samples and stained with 4,6-diamidino-2-phenylindole by the single-step detergent method (19).

Results

In order to detect Rb-LOH, we used a version of the PCR-LOH assay described previously (5). DNA was amplified with primers flanking an XbaI RFLP within intron 17 (22). PCR products were digested with XbaI and electrophoresed on 3% agarose gels. The amplification product was hybridized with a 32P-labeled 400-bp EcoRI fragment from a p53 gene probe. The sizes of the hybridization bands were verified by Southern blotting and hybridization. Southern blots were probed with a XbaI D32971, and the Department of Veterans Affairs.

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Nuclei were then sorted according to DNA content on a Coulter Epics cell sorter, and high molecular weight DNA was obtained from the aneuploid nuclear preparation (50,000 nuclei/specimen) as described previously (20).

Polymerase Chain Reaction. DNA was extracted as outlined (5, 18) and PCRs were performed using reagents as described (5). Three different primer sets were used in separate amplifications (Table 1). PCRs using frozen section and paraffin embedded tissue DNA were performed using 50 and 250 ng genomic DNA, respectively. Reagent amounts, annealing temperatures, and cycle times were optimized for each primer set. PCR products were digested with restriction enzymes and run on polyacrylamide gels as described (5).

DNA Blotting and Hybridization. Purified genomic DNA (10-13 µg) was digested overnight with 100 units of MspI (New England BioLabs). Digested DNA samples were precipitated and electrophoresed overnight in 0.8% agarose/1X TBE gels. Southern blotting using RFLP probe E18 (21) was performed as outlined (5).

Results

In order to detect Rb-LOH, we used a version of the PCR-LOH assay described previously (5). DNA was amplified with primers flanking an XbaI RFLP within intron 17 (22). PCR products were digested with XbaI and electrophoresed on 3% (primer set 1) or 7% (primer set 2) polyacrylamide gels. Tumor DNA demonstrating Rb-LOH showed a diminished signal in either the uncut band or the two cut bands. An example is shown in Fig. 1A. In most tumors loss was not complete, probably because of nontumor cells in the specimen. However, in 8 cases where flow cytometrically sorted tumor nuclei were directly compared to grossly dissected portions of the same cancer, Rb-LOH was consistently more complete (Fig. 2). In 3 of these cases, equivocal results from dissected tissue became unequivocally positive after flow sorting. These findings were not surprising, since aneuploid populations constituted as little as 20% (and up to 90%) of the total cells present in a sorted
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Fig. 1. LOH demonstrated by PCR. N, normal tissue; T, tumor. DNA sizes are indicated in base pairs. In A, DNA was amplified using PCR primer set 1 (Table 1; Ref. 23) flanking a XbaI RFLP site in intron 17 of the Rb gene. A 945-base pair amplicon was created. PCR products were digested with XbaI and run on an agarose gel. The cut allele resulted in 630- and 315-base pair bands. As shown, the intensity of the cut bands is decreased in the tumor, loss is not total, probably because of contaminating normal cells in the tumor mass. B, LOH at the intron 20 variable number of tandem repeats site shown using primer set 3 (Table 1; Ref. 24). The upper allele is partially lost in the tumor. These products were run on polyacrylamide gels. Ordinate, base pairs.

Fig. 2. LOH using sorted nuclei as DNA source. T, DNA from aneuploid nuclei of tumor; N, DNA from normal tissue. reactions were performed as in Fig. 1. Ap, base pairs. Polyacrylamide gel.

tumor sample. As with Southern-based assays, LOH was assumed when the allele:allele ratio in tumor DNA was visibly different from the ratio in matching normal DNA; densitometry was not performed.

Since the RFLP amplified by the two intron 17 primer sets is homozygous in 52% of the population (22), a large number of our samples were uninformative at this site. Therefore, we utilized primer set 3, flanking a variable number of tandem repeats polymorphic site in intron 20 of Rb (Refs. 23 and 24; Fig. 1B). This polymorphism increased our overall informativity rate to 66% (40 of 61). The findings at intron 20 matched those at intron 17 in 12 of 12 individuals heterozygous at both loci.

Using all three primer sets, we detected Rb-LOH in 19 of 40 (48%) informative tumors (Table 1); 14 of 26 squamous cell carcinomas (54%) and 5 of 14 adenocarcinomas (36%) showed Rb-LOH. Five of the tumors assayed by PCR were also analyzed with Southern blotting using probe 1E8, which hybridizes to chromosome 13q on the telomeric side of Rb (21). This probe showed LOH in 3 of 3 informative cases; 2 additional cases were homozygous. All 3 cases with LOH by Southern analysis also exhibited Rb-LOH by PCR analysis. An example of Southern hybridization is shown in Fig. 3.

Discussion

There is a growing list of malignancies characterized by frequently aberrant Rb, including osteosarcoma, soft tissue sarcoma, leukemia, lymphoma, and breast, ovarian, endometrial, lung, and prostate cancers (7–16). We have shown that loss of heterozygosity of Rb is common in esophageal cancer.

We did not detect Rb-LOH in approximately one-half of the samples evaluated. However, our study may have underestimated the true prevalence of Rb-LOH in esophageal cancer. In some tumors that were not cell sorted, LOH could have been masked by large admixtures of nontumor cells or large subpopulations of heterozygous malignant cells from a different clone within the same tumor. Comparisons of flow-sorted versus unsorted tumors support this possibility. Moreover, microdeletions involving only a few exons of Rb have been reported; if such deletions occurred without affecting introns 17 or 20, they would have remained undetected by our assays (16). Alternatively, it is possible that Rb-LOH occurs in a subset of esophageal cancers or that both copies of the gene become inactivated through mechanisms other than allelic loss.

In contrast to the current study, Wagata et al. (4) found Rb-LOH in only 1 of 8 (13%) and chromosome 13q LOH in only 3 of 16 (19%) unmicrodissected esophageal carcinomas. Although the discrepancy could be due to differences in patient

Table 1 Results of PCR-LOH* and Southern blot analyses

<table>
<thead>
<tr>
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<th>% of Informativity (heterozygotes/total cases)</th>
<th>% of LOH (LOH cases/informative cases)</th>
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<tbody>
<tr>
<td>Squamous cell carcinomas</td>
<td>63 (26/41)</td>
<td>54 (14/26)</td>
</tr>
<tr>
<td>Adenocarcinomas</td>
<td>70 (14/20)</td>
<td>36 (5/14)</td>
</tr>
<tr>
<td>Total</td>
<td>66 (40/61)</td>
<td>48 (19/40)</td>
</tr>
</tbody>
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* Primer sets used in PCR-LOH assays were as follows. Set 1, intron 17 XbaI RFLP, 945 base pairs: upstream, TTCAATGAGAAGAAACATGG; downstream, GCAATGCAAAACATAAGTT. Set 2, intron 17 XbaI RFLP, 190 base pairs: upstream, CTGAGTCCCACCCCTAGAGCTAGACATCCACGCAC. Set 3, intron 20 variable number of tandem repeats, 260–300 base pairs: upstream, CTCCTCCTGACTTATCTT; downstream, AATTACAGGTGTTGTTG. Information regarding primer sets 1 and 3 was originally reported by McGee et al. (22) and Yandell et al. (23), respectively.
If Rb inactivation plays a role in esophageal cancer, an increased incidence of the disease in hereditary retinoblastoma survivors might be expected. However, in a survey of the literature, we found only one retinoblastoma survivor who later developed esophageal cancer (28). This is not surprising. Li-Fraumeni syndrome is characterized by germline mutations in another tumor suppressor gene, p53 (29). However, despite the high frequency of p53 mutations in sporadic esophageal cancer (6), Li et al. (30) reported no cases of esophageal cancer in a survey of 119 cancers in 24 Li-Fraumeni syndrome families.

Concurrent abnormalities of Rb and other tumor suppressor genes, such as p53, have been described in human soft tissue sarcomas (12) and human cervical carcinoma cell lines (13). It is possible that inactivation of these other genes occurs cooperatively with Rb-LOH in esophageal cancer; further studies to evaluate this possibility are needed.

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References
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