17p Allelic Deletions and p53 Protein Overexpression in Barrett's Adenocarcinoma

Patricia L. Blount, Stig Ramel, Wendy H. Raskind, Rodger C. Haggitt, Carissa A. Sanchez, Patrick J. Dean, Peter S. Rabinovitch, and Brian J. Reid

ABSTRACT

Barrett's esophagus is a condition in which the stratified squamous epithelium of the esophagus is replaced by metaplastic columnar epithelium that predisposes to the development of esophageal adenocarcinoma. Allelic deletions of 17p and alterations of p53 including elevated p53 protein levels have been observed in many different tumors. To investigate the presence of 17p allelic deletions and p53 protein overexpression in Barrett's adenocarcinomas, we have combined the use of restriction fragment length polymorphism analysis, multiparameter flow cytometry, and DNA content cell sorting. The combined use of these methodologies permits the purification of aneuploid tumor cells for restriction fragment length polymorphism analysis of 17p allelic deletions and the evaluation of p53 protein expression by multiparameter flow cytometry in the same aneuploid tumor cell populations. We analyzed 15 aneuploid populations and one tetraploid population from 13 Barrett's adenocarcinomas for 17p allelic deletions and p53 protein overexpression to determine whether both of these alterations are involved in carcinogenesis in Barrett's esophagus. Twelve of 13 tumors (92%) had 17p allelic deletions, and 8 of 13 tumors (62%) had p53 protein overexpression. Eight of the 12 tumors (67%) with 17p allelic deletions also had p53 protein overexpression. These data indicate that both 17p allelic deletions and p53 protein overexpression are frequently involved in carcinogenesis in Barrett's esophagus.

INTRODUCTION

Barrett's esophagus is a condition in which the normal stratified squamous epithelium of the esophagus is replaced by metaplastic columnar epithelium. It develops as a complication in approximately 10–12% of patients with chronic gastroesophageal reflux and predisposes to the development of esophageal adenocarcinoma (1, 2). During the 1970s and 1980s, the incidence of Barrett's esophagus associated adenocarcinoma increased more rapidly than any other cancer in the United States (3, 4).

Barrett's adenocarcinoma develops by a multistep process in which the specialized metaplastic columnar epithelium progresses to dysplasia and eventually to carcinoma (5). The development of Barrett's adenocarcinoma is associated with the appearance of aneuploid cells with DNA content and karyotypic abnormalities (6–10). In one series, 95% of Barrett's adenocarcinomas had aneuploid or tetraploid cell populations (8). However, relatively little is known concerning the genetic alterations involved in neoplastic progression in Barrett's esophagus. ras gene mutations have been sought but not found (11). Recently, p53 allelic deletions have been reported in three Barrett's adenocarcinomas (12).

The finding of a high prevalence of allelic deletions at a specific locus suggests the existence of a tumor suppressor gene at or near that locus. Detection of allelic deletions requires relatively pure populations of tumor cells that have been separated from stromal cells. This enrichment is usually accomplished by cryostat sectioning (13), but in some upper gastrointestinal adenocarcinomas, this may be difficult because of abundant nonneoplastic stromal cells (14, 15).

There is a high prevalence of 17p allelic deletions in many human tumors including colon, breast, bladder, lung, liver, and sarcomas (13, 16–20). In the colon, >75% of carcinomas have 17p allelic deletions, and the region commonly deleted includes 17p13.1 (21), the location of the p53 gene (22). Many tumors with 17p allelic deletions including colon, lung, breast, and brain have p53 mutations in the remaining 17p allele (23, 24), a finding consistent with the two-hit model proposed by Knudson (25) for tumor suppressor genes.

The wild-type p53 protein has a short half-life and is present at low levels in the nuclei of normal cells (26). Overexpression of the p53 protein, however, has been demonstrated in several different human solid tumors by immunohistochemistry or multiparameter flow cytometry. For example, 54%-56% of colon, lung, and breast carcinomas have p53 protein overexpression (27–30). Although the molecular basis for p53 protein overexpression has not been determined for all human malignancies, overexpression of p53 protein in human colon, breast, and lung carcinomas has been shown to be the result of p53 gene mutations (29, 31–34). Although many somatic p53 mutations result in p53 protein overexpression (35), p53 protein levels are normal in cultured fibroblasts from patients with the Li-Fraumeni syndrome, in which p53 mutations are inherited (36).

We have combined RFLP analysis, multiparameter flow cytometry, and DNA content cell sorting to investigate the presence of 17p allelic deletions and p53 protein overexpression in aneuploid tumor cells from Barrett's adenocarcinomas. Cell sorting permits the purification of aneuploid tumor cells for RFLP analysis of allelic deletions by eliminating nonneoplastic diploid stromal cells.

MATERIALS AND METHODS

Tissue Preparation. Thirteen Barrett's adenocarcinomas were obtained from 13 esophagectomy specimens at the time of surgery, placed in minimal essential media (Gibco, Gaithersburg, MD) with 10% dimethyl sulfoxide and 5% fetal calf serum on ice, and frozen immediately at −70°C until processed. Two of the carcinomas were intramucosal, 5 submucosal, 1 invasive into the muscularis propria, 2 invasive into the adventitia, and 3 metastatic. Nuclei were isolated from tumor samples and stained with DAPI (Accurate Chemical and Scientific Corp., Westbury, NY) by the single-step detergent technique, as previously described (6). The nuclei were sorted according to DNA content on an Ortho 50/2150 cell sorter for RFLP analysis. If 2 aneuploid cell populations were present in a tumor sample, then both were sorted for specific...
the sorted nuclei and normal tissue by lysis in SDS-proteinase K preparation contained 60,000–150,000 nuclei for DNA extraction.

DNA Extraction. High molecular weight DNA was obtained from the sorted nuclei and normal tissue by lysis in SDS-proteinase K followed by phenol-chloroform extraction and ethanol precipitation.

Allelic Deletion. The entire extracted DNA sample from each sorted nuclear preparation, as well as 1–2 μg of DNA from the corresponding normal tissue, was digested in a total volume of 30 μl at 37°C overnight with the restriction endonuclease Hinfl in buffer supplied by the manufacturer (Boehringer Mannheim Corp., Indianapolis, IN). Each aneuploid tumor DNA sample, the corresponding normal DNA, and, when possible, the diploid stromal DNA from the tumor were electrophoresed in adjacent lanes in 1.5% agarose gel in a Wide Mini-Sub DNA Electrophoresis Cell (Bio-Rad Laboratories, Richmond, CA) and transferred to a Zeta-probe blotting membrane (Bio-Rad) by Southern transfer. Probes pYNZ22 and p144D6 (American Type Culture Collection, catalogue numbers 57574 and 59868) detect variable number tandem repeat sequences on 17p (37), which, when used together, result in a combined heterozygosity of 95% (23, 24). The probes were labeled with [32P]dCTP by random primer extension (38). Hybridization was done in 0.5% Blotto, 2× saline sodium phosphate EDTA (1× SSPE = 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4 + 1 mM EDTA) 1% SDS, 0.5 mg/ml sheared salmon sperm DNA, and 10% dextran sulfate overnight at 65°C. Serial washes were at room temperature in 2× SSC 1× SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 2× SSC-0.5% SDS, and 0.5× SSC-0.1% SDS, followed by a final wash at 50°C in 0.1× SSC-0.1% SDS. The membrane was exposed to X-ray film at −70°C. Allelic deletion was detected by absence of one of the alleles present in the DNA from the corresponding normal tissue.

Multiparameter Flow Cytometric Assessment of p53 and DNA Content. Tissue samples were minced in buffer containing 146 mM NaCl, 10 mM Tris base-1 mM CaCl2-0.5 mM MgSO4-0.05% bovine serum albumin-21 mM MgCl2 and centrifuged at 4°C. The resulting suspension was diluted in Dulbecco's phosphate-buffered saline (Gibco Laboratories Life Technologies, Grand Island, NY) with 10.6 mM MgCl2 and centrifuged at 2000 rpm for 10 min at 4°C. The pellet was resuspended in Dulbecco's phosphate-buffered saline with 10.6 mM MgCl2-10% normal goat serum (Vector Laboratories, Burlingame, CA). The primary monoclonal antibody (anti-p53 gene product) Ab421 (Oncogene Science, Manhasset, NY) was added at a concentration of 1.0 μg/ml to one aliquot and an irrelevant IgG2a antibody (purified myeloma protein, Sigma) was added at the same concentration to a second aliquot as a negative control. Each aliquot was incubated for 45 min on ice. The secondary antibody, a goat anti-mouse IgG conjugated with phycocerythrin (Biomed, Foster City, CA) was added at 50 μg/ml and incubated on ice for 45 min. Dulbecco's phosphate-buffered saline with 10.6 mM MgCl2 was added, and each sample was then centrifuged as above. Nuclei were resuspended in 10 μl/ml DAPI. The samples were resuspended through a 25-gauge needle 7 times before flow cytometric analysis.

Flow Cytometry. Samples were analyzed using an Ortho system 50/2150 cell sorter (Ortho Diagnostic Systems, Inc.). Excitation was achieved with two argon ion lasers, one set at 355–360 nm and the second at 488 nm with phycocerythrin (anti-p53) fluorescence collected at 565–585 nm and DAPI (DNA) fluorescence collected at 400–500 nm. Data were collected and displayed as two related parameters [DAPI (DNA, abscissa) versus phycocerythrin (p53, ordinate)]. Proportions of cells in different subsets of phycocerythrin or DAPI intensity distributions were analyzed using computer software, MULTI-2D (Phoenix Flow Systems, San Diego, CA) written by one of the authors (P. S. R.). The software allowed the X-axis DNA positions of the G1 peak of control and p53-stained histograms to be aligned to compensate for small differences. P53 fluorescence intensity for the cells in the histogram was then analyzed according to a modification of the cumulative subtraction technique of Overton (39). For each X-axis (DNA content) value, the immunofluorescence (Y-axis) values of the control histogram were subtracted from the p53 histogram values, leaving only the positive values representing p53 levels elevated above background. P53 positivity was expressed as the percentage of counts remaining after subtrac-

<table>
<thead>
<tr>
<th>Tumor</th>
<th>17p allelic deletiona</th>
<th>DNA content</th>
<th>17p allelic deletionb</th>
<th>p53c</th>
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<tbody>
<tr>
<td>1 Metastatic</td>
<td>N</td>
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<td>2.8 N</td>
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<td>2 Adenitis</td>
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<td>3.1 N</td>
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<td>5 Metastatic</td>
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<td>13 Muscularis propria</td>
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<td>2.9 N</td>
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* Diploid nuclei that were purified by DNA content cell sorting from each tumor.
* Aneuploid nuclei that were purified by DNA content cell sorting from each tumor.
* p53 protein overexpression.
* Depth of tumor invasion.

Fig. 1. RFLP analysis of allelic deletions of chromosome 17p in Barrett’s adenocarcinomas. A and B, autoradiographs showing pYNZ22 hybridized to DNA digested with restriction enzyme Hinfl; C, D, and E, autoradiographs showing p144D6 hybridized to DNA digested with restriction enzyme Hinfl. In A–D, 17p allelic deletions can be seen in the sorted aneuploid nuclei (lane 4) from each of these tumors, but normal tissue (lane N) and sorted diploid nuclei (lane D) from the tumors retain two 17p alleles. The faint bands seen in lane D of C represent only 64,000 sorted diploid nuclei. In E, normal tissue (lane N) and sorted diploid (lane D) and tetraploid (lane T) nuclei from the tumor all retain two 17p alleles. kb, kilobases.
allelic deletions of 17p and p53 protein overexpression were found in early, as well as advanced, Barrett's adenocarcinomas (Table 1).

Allelic deletion of 17p was not detected in one tumor (tumor

Fig. 3. P53 multiparameter flow cytometric histogram of tumor 5. The tumor has an aneuploid population with a DNA content of 3.4 N that has increased p53 fluorescence relative to the diploid population (2 N) and background fluorescence as revealed by monoclonal antibody 421. The legends for A–C are the same as in Fig. 2.

RESULTS

Allelic Deletion. Thirteen of 14 (93%) Barrett's adenocarcinomas evaluated were informative for either pYNZ22 or p144D6, or both. Twelve of 13 informative tumors (92%) had 17p allelic deletions in the aneuploid DNA samples (Fig. 1, Table 1). Two tumor specimens (tumors 7 and 9) had a second

aneuploid population analyzed. In each of these tumors, both aneuploid populations had 17p allelic deletions. One tumor (tumor 12) had an aneuploid population with a 17p allelic deletion and a tetraploid population that retained both 17p alleles. Sorted diploid DNA samples were analyzed from 8 of the 13 tumor specimens; none had 17p allelic deletions.

p53 Protein Overexpression. Each of the 16 aneuploid or tetraploid cell populations from the 13 tumors analyzed for 17p allelic deletions were evaluated for p53 protein overexpression. Control gastric tissue from the same esophagectomy specimens and diploid cell populations from the tumor samples were also analyzed for p53 protein overexpression, and all were negative. Eight of 13 tumors (62%) had p53 protein overexpression in at least one aneuploid cell population (Figs. 2–5, Table 1). Eight of 12 tumors (67%) with one 17p allele had p53 protein overexpression in at least one aneuploid cell population (Table 1).

Allelic deletions of 17p and overexpression of p53 protein were found in early, as well as advanced, Barrett's adenocarcinomas (Table 1).

Fig. 2. P53 multiparameter flow cytometric histograms of tumor 1. A, DNA content illustrated on the X axis on a linear scale. P53 immunofluorescence is illustrated on the Y axis on a logarithmic scale in which 100 channel units represents 3.3 decades. The histogram illustrates that the tumor sample has an aneuploid population with a DNA content of 2.8 N and a diploid population (2 N). The aneuploid population has increased p53 fluorescence relative to the diploid population and to background fluorescence as revealed by the monoclonal antibody Ab421. B, background fluorescence in the same tumor sample stained with a negative control antibody. The axes are the same as in A. Diploid and aneuploid populations are both negative with the control antibody. C, the DNA content flow cytometric representation (X-axis projection) of the data in A. X axis, DNA content; Y axis, relative number of nuclei on linear scales.

Relative to the total counts in the p53 histogram, X-axis (DNA) projections of the subtracted histograms and the p53 histograms were determined, and DNA content was analyzed using the computer program MULTICYCLE (Phoenix Flow Systems). P53 protein overexpression was defined as p53 positivity ≥2 SD above the mean for Barrett's metaplasia negative for dysplasia and 5.5 SD above the mean for gastric fundic gland mucosa.

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Fig. 4. Multiparameter flow cytometric histogram of tumor 4. The tumor has an aneuploid population with a DNA content of 3.5 N that has increased p53 fluorescence relative to the diploid population (2 N) and background fluorescence as revealed by monoclonal antibody 421. In this tumor, there is a slight increase in background fluorescence as revealed by the control antibody in B. The legends for A-C are the same as in Fig. 2.

13) by the probes used in this study. The single aneuploid cell population analyzed from this tumor did not have p53 protein overexpression.

Tumor 9 had two aneuploid cell populations analyzed; both aneuploid populations had the deletion of one 17p allele, but only one of the populations had p53 protein overexpression. The difference in the p53 protein expression in these 2 aneu- ploid populations was not due to sample handling or variability in the assay because the tumor sample analyzed for p53 protein overexpression contained both aneuploid peaks in the same multiparameter histogram.

DISCUSSION

RFLP analysis has been used to detect sites of allelic deletion that may harbor tumor suppressor genes in many human cancers (12, 13, 16-20), but in some upper gastrointestinal adenocarcinomas abundant nonneoplastic stromal cells may obscure the detection of allelic deletions (14, 15). In the present study, we used DNA content cell sorting to purify aneuploid populations of cells from Barrett's adenocarcinomas for RFLP analysis of 17p allelic deletions. By combining RFLP analysis, multiparameter flow cytometry, and DNA content cell sorting, we were able to investigate 17p allelic deletions, p53 protein overexpression, and DNA content abnormalities in the same populations of tumor cells.

We detected a 92% prevalence of 17p allelic deletions in Barrett's adenocarcinoma. Allelic deletions of 17p were found in early as well as advanced adenocarcinomas. These 17p allelic deletions were found only in the sorted aneuploid populations of the tumors; sorted diploid nuclei from the tumors and control gastric tissue had two 17p alleles. The high prevalence of 17p allelic deletions suggests that there is a tumor suppressor gene on 17p that is involved in the pathogenesis or progression of Barrett's adenocarcinoma. To determine whether or not this 17p tumor suppressor gene is p53 will require evaluation of the remaining 17p allele for p53 mutations. It is of note, however, that there is also a high prevalence of p53 protein overexpression in the aneuploid tumor cell populations of Barrett's adenocarcinomas but not in diploid cells from the tumors and control gastric tissue. Although p53 protein overexpression has
been associated with p53 mutations in some human cancers (29, 31–34), further work will be required to determine its molecular basis in Barrett's adenocarcinoma.

Although we detected p53 overexpression in 62% of Barrett's adenocarcinomas in this study, the multiparameter flow cytometric assay that we used has limitations. Tumors that produce a p53 protein that is not detected by monoclonal antibody Ab 521 would be negative by this assay (30). This assay would also not detect p53 protein overexpression in tumors in which the p53 protein is confined to the cytoplasm, although recent reports suggest that this is uncommon (30, 33). Furthermore, the multiparameter flow cytometric assay, like immunohistochemistry, can detect p53 protein expression relative to controls and to background but cannot determine the half-life or exact quantity of p53 protein.

In summary, we have used RFLP analysis, multiparameter flow cytometry, and DNA content cell sorting to demonstrate that aneuploid cell populations in Barrett's adenocarcinomas have a high prevalence of 17p allelic deletions and p53 protein overexpression. Because a cell sorter can be used to purify tissues and to background but cannot determine the half-life or exact quantity of p53 protein.

ACKNOWLEDGMENTS
We thank Karen Stephens and Robert J. Livingston for technical advice and Mark Matsushita for technical assistance.

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