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17p (p53) allelic losses, 4N (G_2/tetraploid) populations, and progression to aneuploidy in Barrett's esophagus

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ABSTRACT **Increased 4N (G_2/tetraploid) cell populations have been postulated to be genetically unstable intermediates in the progression to many cancers, but the mechanism by which they develop and their relationship to instability have been difficult to investigate in humans *in vivo*. Barrett's esophagus is an excellent model system in which to investigate the order in which genetic and cell cycle abnormalities develop relative to each other during human neoplastic progression. Neoplastic progression in Barrett's esophagus is characterized by inactivation of the p53 gene, the development of increased 4N (G_2/tetraploid) cell fractions, and the appearance of aneuploid cell populations. We investigated the hypothesis that patients whose biopsies have increased 4N (G_2/tetraploid) cell fractions are predisposed to progression to aneuploidy and determined the relationship between inactivation of p53 and the development of 4N abnormalities in Barrett's epithelium. Our results indicate that increased 4N (G_2/tetraploid) populations predict progression to aneuploidy and that the development of 4N abnormalities is interdependent with inactivation of the p53 gene in Barrett's esophagus *in vivo*.**

Cancer arises through a multistep process that is characterized by genetic instability, loss of cell cycle regulation, and the evolution of clones of cells with accumulated genetic errors (1–4). Aneuploid cell populations, increased 4N (G_2/tetraploid) cell fractions, and inactivation of p53 by mutation and 17p allelic loss are common abnormalities found in many human neoplasms, including adenocarcinomas of the esophagus (5–10). Barrett's esophagus is an excellent model system in which to investigate the relationships between genetic and cell cycle abnormalities in a human premalignant condition *in vivo* (9, 10). Patients with Barrett's esophagus have a 30- to 125-fold increased risk of developing esophageal adenocarcinoma (11, 12). Therefore, patients are monitored by periodic endoscopic biopsy surveillance, which permits prospective investigation of genetic and cell cycle abnormalities that develop during human neoplastic progression (4, 10, 13). Furthermore, in addition to cancer, surgical specimens frequently contain premalignant epithelium, permitting the investigation of multiple stages of neoplastic progression in the same esophagectomy specimen (14, 15).

In previous investigations, p53 mutations, 17p allelic losses (presumably targeting p53), increased 4N fractions, and aneuploidy have all been found to develop in premalignant epithelium in Barrett's esophagus (8–10). We have also shown that increased 4N (G_2/tetraploid) fractions and aneuploid cell populations identify a subset of patients who have an increased risk of developing high-grade dysplasia or cancer during prospective follow-up (4). It has been hypothesized that increased 4N (G_2/tetraploid) cell populations may be predisposed to develop aneuploidy and other genetic errors during neoplastic progression (6). However, neither the mechanism by which cells with abnormal 4N fractions develop nor their relationship to the development of aneuploidy have been established in humans *in vivo*. Inactivation of p53 has been shown to be associated with the development of tetraploidy, aneuploidy, and other manifestations of genetic instability *in vitro* and in murine model systems *in vivo* (16–20). It has been shown that inactivation of p53 precedes the development of aneuploidy in Barrett's esophagus, but the relationship between 17p allelic losses and 4N (G_2/tetraploid) cell populations has not been determined in this condition.

We have previously used clonal ordering to determine the relationships among different genetic and cell cycle abnormalities that arise during neoplastic progression in Barrett's esophagus *in vivo* (9, 10, 15, 21). The formalism used in ordering was originally developed to determine the order of genetic events in the yeast cell cycle (22). Any two events, *A* and *B*, of a multistep pathway can be related to each other in one of four ways. (*i*) *A* can consistently precede *B*, e.g., *A* could cause *B* or create a condition that is permissive for *B* to occur. (*ii*) *B* can consistently occur before *A*, indicating that *A* is not required for the development of *B*. (*iii*) *A* and *B* may be interdependent events that occur at the same time, e.g., *A* and *B* may effect the same step. (*iv*) *A* and *B* may occur independently of each other. Using this formalism, we have previously shown that the p53 tumor suppressor gene is inactivated in diploid cells by mutation and 17p allelic loss before the development of aneuploidy or cancer in Barrett's esophagus (21).

In this study, we have used Barrett's esophageal biopsies to investigate the hypothesis that increased 4N (G_2/tetraploid) cell fractions predict progression to aneuploidy, and we have investigated the order in which 4N fractions and 17p allelic losses develop relative to each other in a human premalignant condition *in vivo*.

MATERIALS AND METHODS

Patient Selection and Tissue Acquisition. For determination of the incidence of aneuploidy, 90 patients from the University of Washington Barrett's Esophagus Study Cohort were selected using the following criteria: a minimum of two endoscopies, prospective follow-up of at least 12 months, and no aneuploid populations present at the time of initial endoscopic evaluation. Thirty-four patients were selected to evaluate the relationship between 17p allelic losses and 4N fractions. Seven of these patients were a subset of the 90 patients for whom tissue was available (four had evidence of 4N abnormalities

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Abbreviation: PEP, primer extension preamplification.

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and three did not). Endoscopic biopsies and six surgical specimens from 27 additional patients, 15 of whom had increased 4N fractions and 12 of whom had normal 4N fractions, were also evaluated. Inclusion criteria for these 34 patients were informativity for polymorphisms within or linked to the p53 gene on chromosome 17p and available diploid tissue that could be evaluated by Ki67/DNA content flow cytometric cell sorting. The Barrett's Esophagus Study was approved by the Human Subjects Division at the University of Washington in 1983 and renewed annually thereafter.

Flow Cytometry. Tissue samples were evaluated for 4N (G_2/tetraploid) fractions and aneuploid cell populations by DNA content flow cytometry as previously described (4). Aneuploid and diploid nuclei were sorted using a Coulter model Elite cell sorter and frozen at −70°C until the DNA was extracted using our standard techniques (21).

For analysis of 17p allelic losses in diploid biopsies, Ki67/DNA content multiparameter flow cytometry was performed using a Coulter model Elite cell sorter as previously described (21). Cell cycle intervals and DNA content were determined using the program MULTICYCLE (Phoenix Flow Systems, San Diego) using the aggregate subtraction model as described previously (23). Nuclei with a diploid DNA content were sorted into Ki67-positive and Ki67-negative populations, which permits separation of epithelial cells from normal stromal cells because over 90% of Ki67-positive cells in Barrett's mucosa are epithelial cells (3). In biopsies with sufficient cell numbers, total 4N fractions were sorted along with Ki67-positive and Ki67-negative fractions.

Polymerase Chain Reaction. To perform analyses on small DNA samples, a primer extension preamplification (PEP) protocol was used as previously described (24, 25). This protocol consists of preamplifying genomic DNA from ≈1000 sorted cells using a degenerate pentadecanucleotide primer. DNA from flow cytometrically sorted Ki67-positive samples was analyzed for 17p allelic losses using the ALE3 TP53 pentanucleotide VNTR located in the first intron of the p53 gene as previously described (21). Of the 34 patients, seven were not informative for this marker. In these patients, 17p alleles were assessed using D17S5 (YNZ22) at 17p13.3 (15), a *Msp*I RFLP in In6 of the p53 gene (26), or a *Bst*UI RFLP in Ex4 of p53 (26). DNA was not amplified by PEP when these markers were used. Of 105 DNA samples evaluated for 17p allelic losses, 75 were preamplified using the PEP protocol. Of the PEP samples, 31% were validated with non-PEP DNA and 27% were validated with two independent PEP reactions followed by independent locus-specific PCR. All results were reproduced unambiguously. Constitutive DNA was obtained from fundic gland mucosa of each patient and used as an internal control for each sample analyzed.

DNA Sequencing. DNA sequencing of the p53 gene was performed using the Applied Biosystems *Taq* DyeDeoxy Terminator Cycle Sequencing method according to published methods (27).

Statistical Analysis. A χ^2 test for difference in proportions was used to test for a significant association between increased 4N fractions and aneuploidy. Association of 17p allelic losses with the percent 4N (G_2/tetraploid) was analyzed using the generalized estimation equation (GEE) statistical test, which accounts for the lack of independence between observations of the same individual (28).

RESULTS

Increased 4N (G_2/Tetraploid) Fractions and Progression to Aneuploidy. To investigate the hypothesis that increased 4N (G_2/tetraploid) fractions predispose to progression to aneuploidy, we prospectively evaluated 90 patients whose initial endoscopic biopsies were all diploid and who had multiple endoscopies (range 2 to 14) during at least 12 months of follow-up (mean 51.4; range 13.5 to 121.6 months). In 75 patients, 4N fractions remained normal throughout follow-up, whereas increased 4N fractions were detected in the remaining 15 patients at initial or subsequent endoscopies (Table 1). Of the 75 patients without increased 4N fractions, only eight patients (11%) progressed to aneuploidy; the remaining 67 patients (89%) did not develop aneuploidy during a mean follow-up of 52.0 months. In contrast, 11 of 15 patients (73%) with increased 4N fractions subsequently developed aneuploidy. Progression to aneuploidy occurred relatively quickly after detection of increased 4N fractions (mean follow-up of 17.2 months; range 2.3 to 47.1 months). The presence of increased 4N cell populations is thus strongly associated with the subsequent development of aneuploidy ($P < 0.0001$).

4N (G_2/Tetraploid) Cell Populations in Diploid Biopsies with and Without 17p Allelic Losses. We have shown previously that 17p allelic losses and p53 mutations develop in diploid cell populations before aneuploidy in Barrett's esophagus (21, 27). As described above, increased 4N cell populations predispose cells to progression to aneuploidy. Therefore, we investigated the prevalence of 17p allelic losses in 105 nonaneuploid biopsies from 34 patients with or without increased 4N fractions in Barrett's esophagus (Fig. 1). The mean 4N fraction was 2.7% in 66 diploid biopsies that retained both 17p alleles compared with 9.5% in 39 diploid biopsies with a 17p allelic loss. The percent of 4N (G_2/tetraploid) fraction had a highly significant association with 17p allelic loss ($P = 0.0001$). Examples of 17p analyses are shown in Fig. 2. To test whether this increased 4N fraction was due to a generalized increase in proliferation that increased the G_2 fractions nonspecifically, we assessed the S phase fractions and detected no difference between biopsies with and without 17p allelic losses (data not shown). To confirm that the 17p allelic losses were associated with inactivation of p53, we sequenced the p53 gene in five patients; all of these patients had somatic p53 mutations in the remaining allele (data not shown). Constitutive tissues remained wild-type with neither p53 mutations nor 17p allelic losses detected.

Change in 4N (G_2/Tetraploid) Cell Populations with Acquisition of 17p Allelic Loss in Individual Patients. The above data indicate a strong association between 17p allelic losses and increased 4N cell populations in Barrett's esophagus, but they do not permit determination of the order in which the two abnormalities developed relative to each other *in vivo*. Therefore, we investigated the change in the 4N (G_2/tetraploid) cell populations with acquisition of a 17p allelic loss in diploid cell populations from 10 informative patients, each of whom had diploid biopsies both with and without 17p allelic losses (Table 2). In all 10 patients, mean 4N fractions increased in association with the detection of 17p allelic losses, rising from a mean of 3.3% in diploid biopsies with two 17p alleles to a mean of 9.3% in diploid biopsies with a 17p allelic loss.

In eight of these 10 patients, sufficient tissue was available to show that the same 17p allele was lost in 2N, 4N, and, when available, aneuploid cell populations, suggesting that the 2N, 4N, and aneuploid cell populations developed by a process of clonal evolution (Fig. 3). To confirm that these cell populations evolved from a common precursor, we sequenced the p53 gene in 2N, 4N, and aneuploid cell populations of one patient. The

Table 1. Prospective development of aneuploidy in Barrett's esophagus

Flow cytometry	No. developing aneuploidy (%)
4N ≤ 6%	8/75 (11)
4N > 6%	11/15 (73)

Prospective development of aneuploid cell populations in 90 patients with ($n=15$) and without ($n=75$) increased 4N (G_2/tetraploid) cell populations.

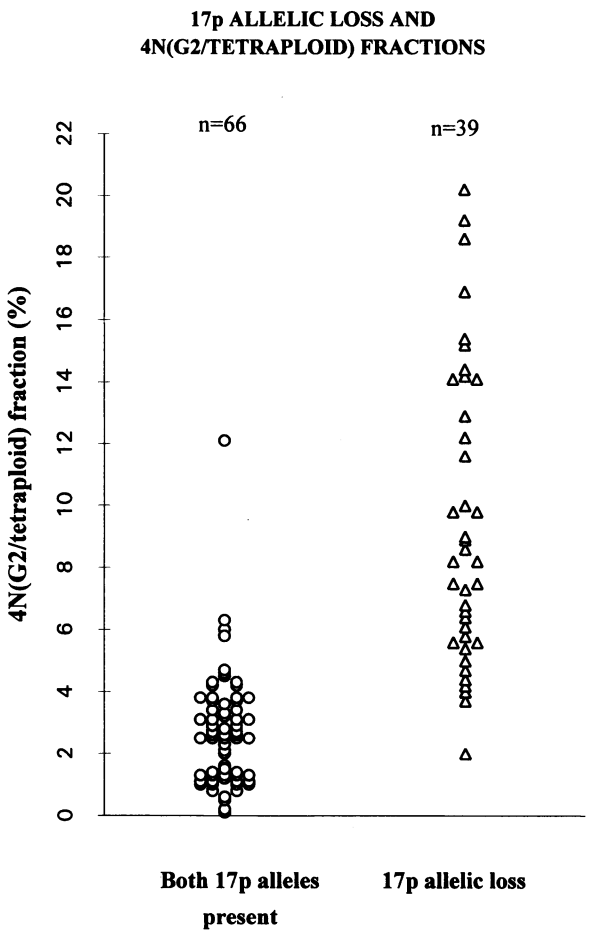

FIG. 1. 17p (p53) allelic losses and 4N (G_2/tetraploid) fractions in 105 diploid biopsies. The mean 4N (G_2/tetraploid) fraction increased from 2.7% in 66 cell populations that retained both 17p alleles to 9.5% 39 in cell populations with 17p allelic losses.

same p53 mutation and 17p allelic loss were detected in each of these populations, indicating that the 2N, 4N, and aneuploid cells developed by a process of clonal evolution.

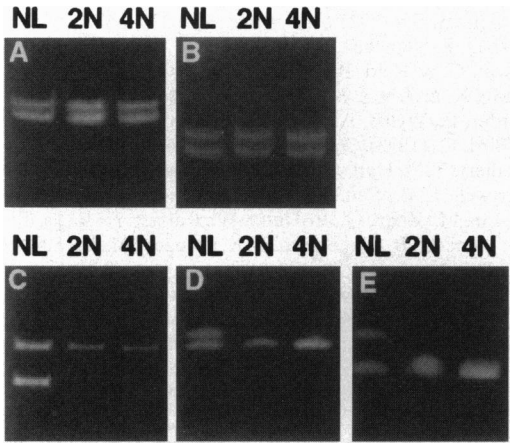

FIG. 2. Nonaneuploid biopsies were sorted into Ki67-positive 2N and total 4N fractions and the number of 17p alleles was determined relative to constitutive normal (NL) tissue. (*A* and *B*) Two patients with normal 4N (G_2/tetraploid) fractions (1.0% and 3.8%, respectively) that retain both 17p alleles. (*C–E*) 2N and 4N fractions that have 17p allelic loss. The 4N fractions for the biopsies illustrated in *C–E* are 20.2%, 12.9%, and 15.2%, respectively.

Table 2. 17p allelic losses and mean 4N fractions in Barrett's esophagus

Patient	Both 17p alleles present	17p allelic loss
1	1.3% (2)	10.8% (3)
2	3.5% (2)	9.2% (3)
3	4.7% (1)	11.4% (3)
4	2.7% (5)	11.9% (3)
5	1.0% (1)	4.6% (3)
6	1.8% (5)	8.6% (1)
7	3.8% (1)	10.9% (2)
8	2.4% (2)	12.9% (1)
9	3.2% (5)	7.2% (2)
10	5.2% (8)	7.8% (4)
Mean (total)	3.3% (32)	9.3% (25)

Change in mean 4N (G_2/tetraploid) cell fraction with the acquisition of a 17p allelic loss in 10 patients. The no. of biopsies is in parentheses.

DISCUSSION

Increased 4N (G_2/tetraploid) cell populations have been observed in a large number of human cancers and precancerous conditions; it has been postulated that these populations are predisposed to the development of additional genetic abnormalities during neoplastic progression (6). However, the mechanism by which increased 4N cell populations develop and their association with genomic instability during prospective follow-up have not been established previously in humans *in vivo*. Previous studies in human colorectal carcinoma have reported p53 immunostaining, presumably detecting mutant p53 protein, in 2N cell populations before the development of 4N abnormalities; the investigators suggested that inactivation of p53 may lead to endoreduplication in humans *in vivo* (29). In addition, several investigators have demonstrated that inactivation of p53 in mouse cells is associated with a diploid → tetraploid → aneuploid sequence of genomic instability (19, 20). Our results suggest that inactivation of p53 and increased 4N fractions are detected as interdependent events during neoplastic progression of Barrett's epithelial cells and that increased 4N (G_2/tetraploid) fractions are predictors of subsequent development of aneuploidy. Because detection of 17p allelic losses requires the cells to grow into a clone, it is possible that inactivation of p53 and the development of increased 4N fractions may have a dependent relationship at the molecular level that cannot be resolved at the level of human biopsies *in vivo*. Nevertheless, our results indicate a strong association between 17p allelic losses, the development of increased 4N fractions, and the progression to aneuploidy in humans *in vivo*.

Cell cycle checkpoints can prevent genetic instability by causing cell cycle arrest after exposure to genotoxic injury or failure to complete previous cell cycle events (30, 31). Inactivation of p53 might lead to increased 4N (G_2/tetraploid) cell populations by either of two checkpoint mechanisms. First, p53 mediates a checkpoint in G_1 as part of a signal transduction pathway that responds to DNA strand breaks and prevents entry into S phase (32). If this G_1 checkpoint is inactivated by

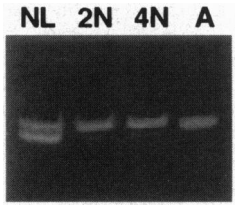

FIG. 3. 17p allelic loss with AL3 (p53 VNTR) in 2N, 4N, and 3.2N aneuploid (A) cell populations from a single patient. The same 17p allele is lost in all samples relative to the constitutive (NL) tissue.

loss of the p53 gene, then cells can replicate the unrepaired DNA, leading to double strand breaks that cause arrest at a subsequent checkpoint in G_2 (4N DNA content) (33). If this model is correct, then either the mammalian G_2 checkpoint adapts if the DNA damage is not repaired, as has been observed in *Saccharomyces cerevisiae,* or the loss of p53 lowers the threshold for adaptation because we and others have observed that the 4N cells progress to aneuploidy (19, 31, 34). Recent investigations suggest that p53 may alter the threshold for G_2 arrest in rodent cells, but it is not known whether this is also the case in human cells (35). Second, in mouse embryonic fibroblasts, p53 has recently been shown to mediate a checkpoint that prevents cell cycle progression when spindle function is disrupted (18, 36). When this checkpoint is inactivated by loss of the p53 gene, cells can continue cell cycle progression without completing chromosome segregation in mitosis, leading to the development of cycling tetraploid cell populations. Although our results do not permit us to distinguish between these two mechanisms in Barrett's esophagus, they suggest that inactivation of p53, with loss of its checkpoint functions, can lead to development of increased 4N fractions and that patients whose biopsies have increased 4N fractions are predisposed to the development of aneuploid cell populations. We have shown previously that patients whose biopsies have increased 4N fractions or aneuploidy are at increased risk of progression to high-grade dysplasia or cancer (4). Our present results indicate that the development of increased 4N fractions is an early abnormality that may be especially useful for the early detection of patients who are at increased risk for progression to aneuploidy and cancer in Barrett's esophagus.

In our study, the actual times at which the subjects first developed increased 4N (G_2/tetraploid) and aneuploid cell populations were not observed. However, we were able to observe whether these events occurred during the interval between endoscopies. The 4N intermediate has been postulated to be unstable (6) and, therefore, may not be detected if the time between endoscopies is lengthy. In our study, the mean interval between endoscopies was 31.1 months (range 12.0–95.6 months) in the eight patients who developed aneuploid cell populations without a 4N abnormality. In contrast, in 11 patients with increased 4N fractions, the mean interval between endoscopies from development of a 4N abnormality to development of aneuploidy was 17.2 months (range 2.3–47.1 months). Thus, it is possible that a 4N abnormality could have been missed during the time between endoscopic evaluations in the eight patients who appeared to progress directly to aneuploidy.

Our results suggest a mechanism for the development of aneuploid cell populations in a human premalignant condition *in vivo*. At an early stage of progression, loss of p53 function leads to inactivation of a cell cycle checkpoint(s), resulting in the development of increased 4N fractions. These 4N (G_2/tetraploid) populations are predisposed to further genomic instability, the evolution of aneuploid cell populations, and progression to cancer.

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