

Cell Proliferation, Cell Cycle Abnormalities, and Cancer Outcome in Patients with Barrett's Esophagus: A Long-term Prospective Study

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Abstract Purpose: Elevated cellular proliferation and cell cycle abnormalities, which have been associated with premalignant lesions, may be caused by inactivation of tumor suppressor genes. We measured proliferative and cell cycle fractions of biopsies from a cohort of patients with Barrett's esophagus to better understand the role of proliferation in early neoplastic progression and the association between cell cycle dysregulation and tumor suppressor gene inactivation.

Experimental Design: Cell proliferative fractions (determined by Ki67/DNA multiparameter flow cytometry) and cell cycle fractions (DNA content flow cytometry) were measured in 853 diploid biopsies from 362 patients with Barrett's esophagus. The inactivation status of *CDKN2A* and *TP53* was assessed in a subset of these biopsies in a cross-sectional study. A prospective study followed 276 of the patients without detectable aneuploidy for an average of 6.3 years with esophageal adenocarcinoma as an end point.

Results: Diploid S and 4N (G₂/tetraploid) fractions were significantly higher in biopsies with *TP53* mutation and loss of heterozygosity. *CDKN2A* inactivation was not associated with higher Ki67-positive, diploid S, G₁, or 4N fractions. High Ki67-positive and G₁-phase fractions were not associated with the future development of esophageal adenocarcinoma ($P = 0.13$ and $P = 0.15$, respectively), whereas high diploid S-phase and 4N fractions were ($P = 0.03$ and $P < 0.0001$, respectively).

Conclusions: High Ki67-positive proliferative fractions were not associated with inactivation of *CDKN2A* and *TP53* or future development of cancer in our cohort of patients with Barrett's esophagus. Biallelic inactivation of *TP53* was associated with elevated 4N fractions, which have been associated with the future development of esophageal adenocarcinoma.

Increased cellular proliferation and dysregulation of the cell cycle have been reported in advancing histologic grades of neoplastic progression in a large number of cross-sectional studies (1–4). Advances in basic science over several decades support the hypothesis that these changes are due to mutation,

loss, or inactivation of cell cycle control genes. The initial genetic model of the eukaryotic cell cycle reported that a regulatory element, called START in the yeast *Saccharomyces cerevisiae*, controlled the transition from G₁ to S phase (5). This regulatory element was subsequently shown to be evolutionarily conserved, and similar G₁-S phase controls were identified in mammalian species, including humans (6). The potential importance of G₁-S regulation in human neoplastic progression became clearer when tumor suppressor genes, such as *CDKN2A* (*p16*) and *TP53* (*p53*), were identified, and well-designed molecular biological studies in model systems and organisms elucidated mechanisms of tumor suppression that included control of the G₁-S phase transition (7–10). These tumor suppressors could be inactivated by a two-hit mechanism involving loss of heterozygosity (LOH) of one allele and mutation or methylation of the second (11–13). Abnormalities involving *CDKN2A* and *TP53* are among the most commonly reported in human cancers and premalignant neoplasms (14, 15).

Barrett's esophagus is a condition in which the normal esophageal squamous epithelium is replaced by an intestinal metaplasia associated with an increased risk of developing esophageal adenocarcinoma (16). Cell proliferation in Barrett's epithelium is similar to the small intestine, but increased compared with normal esophageal squamous epithelium (17, 18). Proliferation has been measured by a variety of

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Translational Relevance

Increased cellular proliferation has long been hypothesized to be associated with progression to cancer. In this prospective cohort study, we found no association between cellular proliferation (measured using Ki67) and the future development of esophageal adenocarcinoma in cancer-free individuals with Barrett's esophagus. However, we found evidence that high 4N fractions, which had previously been shown to be strongly predictive of the development of esophageal adenocarcinoma, are a result of biallelic inactivation of *TP53*. Cell cycle abnormalities and loss of tumor suppressor genes are common somatic genetic abnormalities in human cancers and are therefore promising biomarkers for predicting neoplastic progression in many premalignant conditions. In this study, we have shown a connection between these two types of biomarkers in human clinical samples.

techniques in Barrett's esophagus, including tritiated thymidine incorporation; immunohistochemical markers, such as Ki67, proliferating cell nuclear antigen, cyclin A, and minichromosome maintenance proteins; DNA content flow cytometry; and multiparameter flow cytometry (17–26). Several, but not all, cross-sectional studies using DNA content flow cytometry, multiparameter flow cytometry, and immunohistochemistry have reported associations between abnormal proliferation/cell cycle fractions and advancing grades of dysplasia (18, 19, 21, 24–27). Similarly, in cross-sectional studies of individual Barrett's esophagus crypts, the total number of proliferating cells seems to increase with progressive grades of dysplasia due to an expansion of the crypt proliferative compartment (22). Two previous studies, one in Barrett's esophagus and one in colonic adenomas, have reported an association between *TP53* abnormalities and elevated 4N fractions (28, 29). However, the effects of loss of these genes on cell proliferation in human diploid biopsies *in vivo* are largely unknown.

The number of prospective studies of proliferative/cell cycle abnormalities as predictors of progression from Barrett's esophagus to esophageal adenocarcinoma is much smaller. One study reported increased 4N fraction (and to a lesser extent, S phase) to be associated with progression to esophageal adenocarcinoma in persons with Barrett's esophagus (23). However, no previous prospective cohort study has comprehensively evaluated increased proliferation and cell cycle fractions as candidate predictors of progression from Barrett's esophagus to esophageal adenocarcinoma. In addition, no previous cohort study has comprehensively evaluated associations between proliferation and cell cycle fractions and inactivation of *CDKN2A* and *TP53* to determine whether abnormal proliferation is associated with loss of *CDKN2A* and *TP53* regulation of the transition from G₁ to S phase.

The Seattle Barrett's Esophagus Study is designed around a dynamic prospective cohort whose research participants are being followed to identify risk and protective factors that are associated with progression or lack of progression to esophageal adenocarcinoma (23, 30–33). Here, we report for the first time results of a comprehensive study of proliferative and cell cycle abnormalities in diploid cells as predictors of progression

from Barrett's esophagus to esophageal adenocarcinoma using Ki67/DNA content multiparameter flow cytometry. Ki67 antibody labels nuclei in the G₁, S, G₂, and M phases of the cell cycle, but not in G₀ (quiescent cells; ref. 34). By combining Ki67 labeling and DNA content flow cytometry, it is possible to measure total proliferative fractions as well as individual cell cycle phases, including G₁, S, and G₂-M (21). We tested the associations between total proliferative and cell cycle fractions and progression from Barrett's esophagus to esophageal adenocarcinoma in 276 individuals. To our knowledge, this is the first prospective cohort study of cancer risk prediction based on cellular proliferation in cancer-free individuals. We were also able to compare the proliferative/cell cycle abnormalities directly with *CDKN2A* and *TP53* status in the same biopsies by Ki67/DNA content multiparameter flow sorting for LOH, mutation, and methylation detection. This cross-sectional analysis of biopsies from 362 individuals is, to our knowledge, the first to comprehensively investigate diploid cell proliferative/cell cycle fractions and *CDKN2A* and *TP53* abnormalities in the same biopsies in humans.

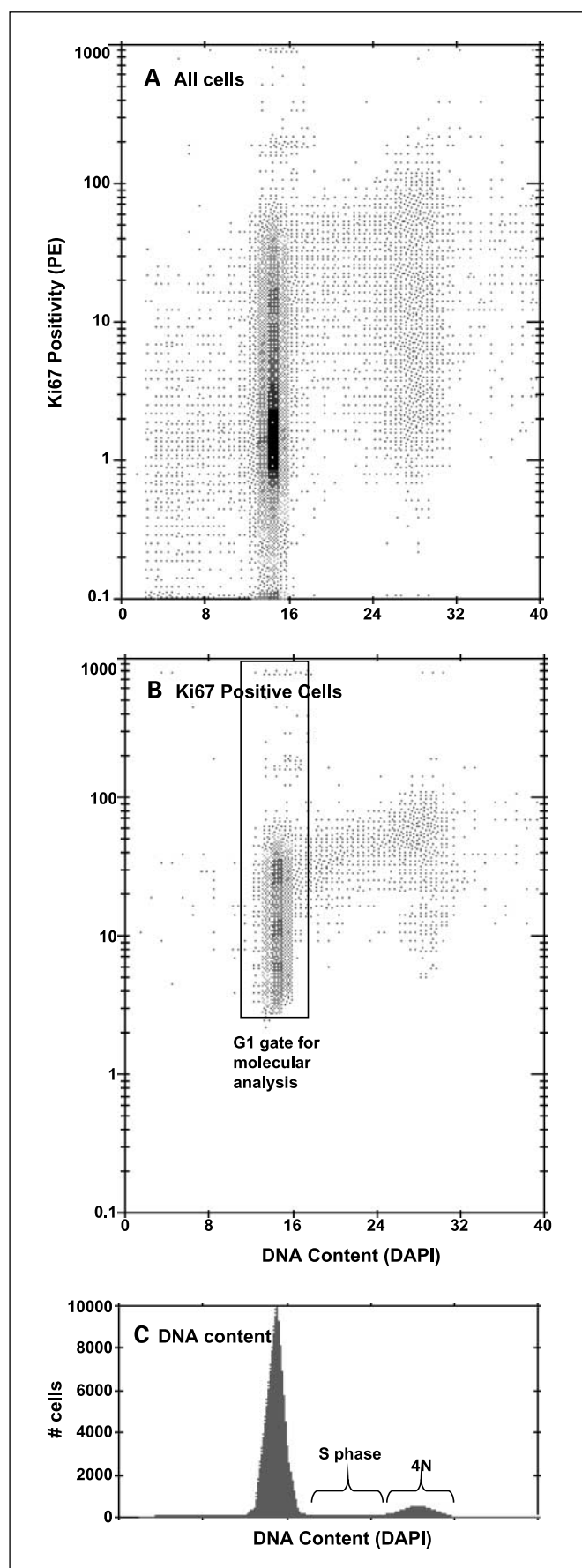
Materials and Methods

Study subjects and tissue acquisition. The 362 participants in the study (Table 1) were enrolled in the Seattle Barrett's Esophagus Study between 1983 and 1999. Participants with esophageal adenocarcinoma at baseline were excluded from analysis. Baseline biopsies were obtained between 1989 and 2003. Biopsies from 214 of the participants were evaluated for *CDKN2A* mutation, 192 for *TP53* mutation, 245 for 9p LOH, 241 for 17p LOH, and 96 for *CDKN2A* methylation. The 276 participants were followed from 89 d to 12.1 y. The Seattle Barrett's Esophagus Study was approved by the Human Subjects Division of the University of Washington in 1983 and renewed annually thereafter with reciprocity from the Fred Hutchinson Cancer Research Center Institutional Review Board from 1993 to 2001. Since 2001, the study has been approved by the Fred Hutchinson Cancer Research Center Institutional Review Board with reciprocity from the University of Washington Human Subjects Division.

Endoscopic biopsy protocols used in the Seattle Barrett's Esophagus Study have been published previously (23, 30). Four quadrant biopsies for histology were taken every 1 cm (for patients with high-grade dysplasia) or every 2 cm (for patients without high-grade dysplasia) at intervals ranging from every 6 mo (for high-grade dysplasia) to 3 y, as described previously (23, 30). Endoscopic biopsies every 2 cm for flow

Table 1. Participant information at the time of the baseline visit (362 participants)

	No. persons (%)
Gender	
Male	293 (80.9)
Female	69 (19.1)
Age (y)	
26-55	108 (29.8)
55-70	142 (39.2)
70+	112 (30.9)
Follow-up	
None	86 (23.8)
89 d-2 y	40 (11.0)
2-5 y	71 (19.6)
5-10 y	114 (31.5)
10+ y	51 (14.1)



cytometry and molecular studies were placed into media with 10% DMSO within 15 s and held on ice until frozen and stored at -70C.

We included all cancers that developed subsequent to the baseline evaluation so that accurate risk stratification models can be developed based on findings at a single baseline endoscopy. All participants had at least one biopsy evaluated for biomarkers every 2 cm in the Barrett's segment regardless of the histologic diagnosis. For the prospective study of cancer outcome, we included only the biopsies obtained for biomarker analysis by the standard one biopsy per 2 cm protocol to avoid sampling bias.

Ki67/DNA content multiparameter flow cytometry and sorting. Frozen endoscopic biopsies ($n = 853$) were prepared for flow cytometry as described previously (19, 35). To ensure accurate detection of 4N DNA content abnormalities, the suspension of unfixed nuclei from each biopsy was aliquoted into separate microfuge tubes for DNA content flow cytometric analysis and multiparameter Ki67/DNA content cell sorting. The aliquots with 4',6-diamidino-2-phenylindole (10 $\mu\text{g}/\text{mL}$; Accurate Chemical) saturated nuclei for DNA content flow cytometry were never centrifuged and were triturated with a 25-gauge needle immediately before evaluation on the flow cytometer to prevent clumping of nuclei that will increase the 4N population as an artifact. DNA content analysis was done using MultiCycle software with a standardized peak versus area gate to exclude residual doublets and with "sliced nucleus" background correction. In accordance with published guidelines (36), histograms containing <10,000 events or debris in excess of 20% were not considered adequate for S-phase analysis. Two of the authors (C.A.S. and P.S.R.) interpreted flow cytometric histograms independently, with disagreements resolved by joint review of the histogram. See Fig. 1C for a representative DNA content histogram.

Ki67/DNA content multiparameter cell sorting was used to purify the proliferating Barrett's esophagus epithelium from nonproliferating G_0 cells into cell cycle fractions including G_1 and 4N ($G_2/\text{tetraploid}$) for molecular studies as previously described (21, 33, 35). Briefly, the nuclei were incubated with directly conjugated Ki67-R-phycoerythrin or isotype control-R-phycoerythrin (DAKO R0840; DAKO reagent no longer available, BD Pharmingen #556027 markets a similar reagent) and 4',6-diamidino-2-phenylindole for DNA content. The antibody protocol includes a centrifugation step and is therefore inadequate for accurate assessment of the 4N fraction. Ki67/DNA content flow cytometry was also used to assess total Ki67-positive proliferative fractions and Ki67-positive G_1 fractions. The Ki67-positive fraction is calculated by bivariate curve subtraction of the negative control from the Ki67-stained cytogram with the program Multicycle (Phoenix Flow Systems) as previously described (21). Samples were sorted on a Beckman Coulter Elite cell sorter. For the molecular analyses used in this study, the Ki67-positive diploid G_1 cells were sorted (Fig. 1B). Data from biopsies with aneuploid fractions were not used in this study.

Microsatellite LOH, DNA sequence, and p16 methylation analyses. Flow-sorted fractions from diploid biopsies were evaluated for 9p21 (*CDKN2A/p16*) and 17p13 (*TP53/p53*) LOH using polymorphic microsatellite markers, as described previously (35, 37-39). DNA was extracted from the Ki67/DNA content sorted cell populations, and whole genome amplification using primer extension preamplification was done as described previously (37) for each sorted fraction and three constitutive controls per participant. LOH status for chromosome 9 was

Fig. 1. Ki67-phycoerythrin and DNA content flow cytometry. *A*, bivariate histogram displaying 4',6-diamidino-2-phenylindole (DAPI) fluorescence on the x-axis (linear) and Ki67-phycoerythrin (PE) fluorescence on the y-axis (logarithmic; 4 decades) of diploid cells in a Barrett's esophagus biopsy. *B*, Ki67-positive proliferating diploid cells, as determined using the bivariate subtraction algorithm (Multicycle). The total Ki67-positive fraction of this sample was calculated to be 35.5%. A representation of the diploid G_1 sorting gate is shown. *C*, a single parameter histogram of DNA content, with S and 4N ($G_2\text{-M}$) cells indicated. The S-phase fraction is 6.5% and the 4N fraction is 9.9%. There was no evidence of a tetraploid cell cycle.

assessed in 526 samples from 245 participants, and that for chromosome 17 was assessed in 521 samples from 241 participants.

DNA was sequenced using BigDye or BigDyeV3 Terminator cycle sequencing (Applied Biosystems) on an ABI 377, 3700, or 3730 DNA sequencer. Wild-type sequences were confirmed using constitutive samples. All mutations were confirmed by at least two independent PCR and sequencing reactions. Evaluation of mutation of exons 5 to 9 of the *TP53* gene was done on 382 flow-purified fractions from 192 participants as described previously (11). Sequence analysis of exon 2 of the *CDKN2A* gene was done on 424 flow-purified fractions from 214 participants as described previously (13).

One hundred sixty-five samples from 96 participants were evaluated for methylation of the *CDKN2A* CpG islands as described previously (13). A subset of these results were previously reported in a cross-sectional study (13). Samples were classified as either positive or negative for *CDKN2A* methylation on the basis of a positive result from methylation-specific PCR done on bisulfite-treated DNA. The requirement for larger amounts of DNA for input into the bisulfite reaction limited the number of samples that could be analyzed by this technique.

Statistical analysis. Univariate proportional hazards models were used to calculate the hazard ratios, 95% confidence intervals (95% CI), and *P* values for the association of Ki67-positive and cell cycle fractions with cancer outcome. The Wilcoxon rank-sum test was used to compare Ki67-positive and cell cycle fractions between sets of biopsies. Linear regression was used to measure the correlation between Ki67 and S-phase fractions. A χ^2 test was used to determine the association among *CDKN2A* and between *TP53* inactivation events. Receiver operating characteristic curves (40) were used to plot the sensitivity and specificity of using various thresholds of S-phase or 4N fractions to discriminate between biopsies with and without *TP53* inactivation. The sensitivity and specificity using all S-phase and 4N fractions observed in our samples were used as thresholds to create the receiver operating characteristic curves. Areas under the curves (AUC), a measure of discriminator accuracy with a range of 0.5 (no discrimination) to 1.0 (perfect discriminator), were computed using the trapezoidal rule. In standard receiver operating characteristic analyses, the output of the classifier is inverted if a classifier performs worse than random. However, because we hypothesize that inactivation of *TP53* increases S and 4N fractions, all specificities and sensitivities are computed in terms of the number of *TP53*-inactivated samples with S or 4N fractions above a threshold. Therefore, we allow our analysis to include classifiers that perform worse than chance. In all analyses, neutral *TP53* mutations were considered equivalent to *TP53* wild-type. The 95% CIs for the AUCs were estimated using the bootstrap (10,000 trials). Statistical analyses were carried out using the R statistical computing language version 2.5.1 (R Foundation for Statistical Computing, Vienna, Austria, 2007).

Results

Of the 276 participants with follow-up (average of 6.3 years, 89 days-12.1 years, total of 1,752 person-years), 29 developed esophageal adenocarcinoma. High S and 4N fractions were significantly associated with future development of esophageal adenocarcinoma ($P = 0.03$ and $P < 0.0001$ modeling the fractions as continuous variables, respectively), whereas high Ki67-positive (the proliferative fraction that comprises cells in G_1 , S, and G_2 -M) and G_1 fractions were not associated with subsequent progression to cancer ($P = 0.13$ and $P = 0.15$, respectively; Table 2). Because cells with the largest cell cycle abnormalities may have the greatest contribution to neoplastic progression, the associations were computed using the biopsy with the maximum Ki67-positive, G_1 -phase, S-phase, and 4N fractions for patients with more than one biopsy. However, estimating the associations using the average values did not affect the results (data not shown). Aneuploid biopsies were excluded from this analysis because aneuploidy is a late event in neoplastic progression and has already been associated with a high risk of progression to esophageal adenocarcinoma in this cohort (23). This allowed us to directly test the hypotheses that proliferative and cell cycle abnormalities are predictors of progression from Barrett's esophagus to esophageal adenocarcinoma that develop as early events due to inactivation of the tumor suppressors *CDKN2A* and *TP53*. Further, the calculation of the diploid S phase is more accurate in diploid biopsies because the software algorithm that calculates the diploid S-phase fractions does not have to account for the overlapping aneuploid cell population.

Although Ki67-positive fractions were not associated with cancer outcome and diploid S-phase fractions were, these two frequently used measures of cellular proliferation were statistically significantly correlated but with a low correlation coefficient in the Barrett's esophagus biopsies ($P < 0.0001$, adjusted $r^2 = 0.12$).

We tested the associations between *CDKN2A* inactivation and cell proliferative and cell cycle fractions. Ki67-positive, S-phase, and 4N fractions were measured in diploid Barrett's esophagus biopsies at the same time as the Ki67-positive G_1 cells were sorted for subsequent examination of the mutation and LOH status of *CDKN2A* and *TP53* and the CpG island methylation status of *CDKN2A*. Eighty-six biopsies without detectable *TP53* inactivation were evaluated for all three

Table 2. Associations between the future development of esophageal adenocarcinoma and Ki67-positive and cell cycle fractions

Index	Outcomes	No. persons	HR (95% CI)*	P
Ki67-positive fraction	29	276	1.02 (0.99-1.05)	0.13
G_1	29	276	1.02 (0.99-1.06)	0.15
S-phase fraction	26	253 [†]	1.16 (1.01-1.32)	0.03
4N fraction	29	269	1.36 (1.26-1.47)	<0.0001

NOTE: The Cox regressions were done using the maximum values per patient obtained from their baseline endoscopies using a 2-cm sampling protocol.

Abbreviation: HR, hazard ratio.

*Hazard ratios and 95% CIs are scaled to indicate increased risk of progression for each 1% increase in Ki67-positive, S phase, or 4N fraction.

[†]S-phase fractions in biopsies from 16 patients could not be accurately estimated.

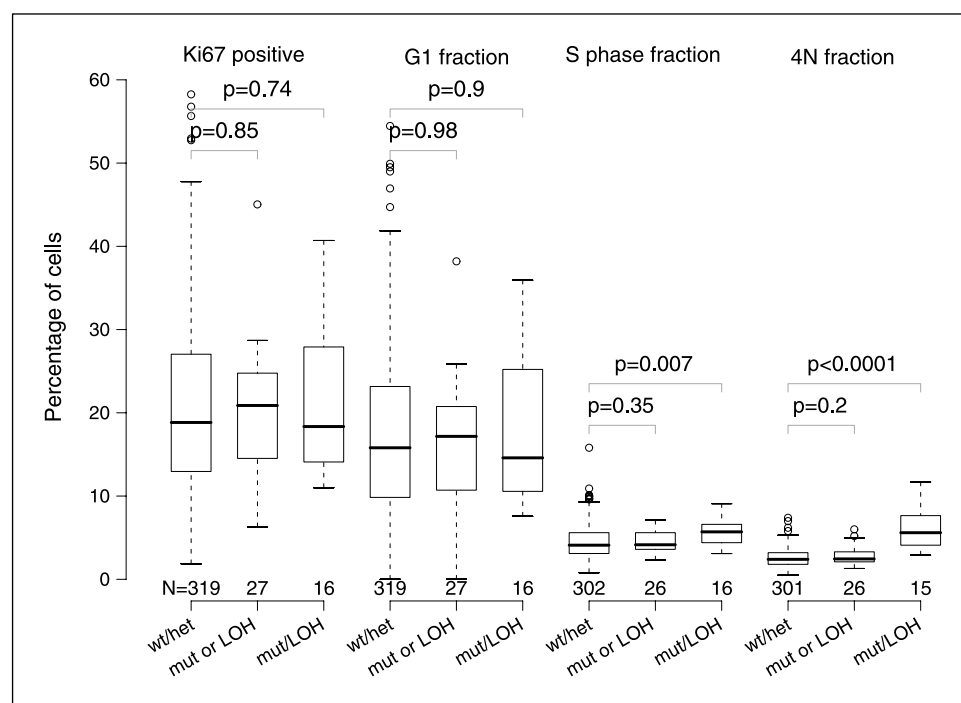


Fig. 2. Ki67-positive, G₁-phase, S-phase, and 4N fractions of biopsies by *TP53* inactivation status. Measurements from biopsies with no *TP53* inactivation (*TP53* wt/17p het), with either *TP53* mutation or 17p LOH (*TP53* mutation or 17p LOH), and with both mutation and LOH (*TP53* mutation and 17p LOH) are summarized. The boxes indicate median and middle two quartiles, the whiskers the 95th percentiles, and the outliers are plotted as circles. The number of samples in each group is printed below each box. The biopsies with no *TP53* inactivation (*TP53*^{+/+}) were compared with the other two groups (*TP53*^{+/-} and *TP53*^{-/-}) using the Wilcoxon rank sum test, and the *P* values are shown on the plot.

mechanisms of *CDKN2A* inactivation. Among these biopsies, there were no significant differences in Ki67 proliferative, G₁, diploid, S phase, or 4N fractions between biopsies with no detectable *CDKN2A* inactivation and those with at least one form of inactivation (*P* > 0.1 for all fractions) or those with two or all three forms (*P* > 0.1). In addition, none of the *CDKN2A* inactivation mechanisms were associated with statistically significantly higher Ki67-positive, G₁-phase, S-phase, or 4N fractions when the mechanisms were analyzed univariately in *TP53*^{+/+} biopsies. *CDKN2A* methylation and 9p LOH co-occurred in biopsies less frequently than expected by chance (*P* = 0.0001, χ^2 test).

S-phase and 4N fractions were significantly higher in diploid biopsies with both *TP53* mutation and 17p LOH than those with no detectable *TP53* alterations (+/+) (*P* = 0.007 and *P* < 0.0001, respectively), whereas they were not significantly different between *TP53*^{+/+} samples and those with only one form of *TP53* abnormality (Fig. 2). *TP53* mutation and 17p LOH co-occurred in biopsies more frequently than expected by chance (*P* = 0.0005, χ^2 test). Analyses of the effects of *CDKN2A* inactivation excluded biopsies with detectable *TP53* alterations to determine the effects of *CDKN2A* inactivation in isolation. Biopsies without detectable *CDKN2A* inactivation could not be excluded in the analyses of *TP53* inactivation because *TP53* inactivation usually occurs in a background of *CDKN2A* inactivation (41).

Because *TP53* abnormalities were significantly associated with high S-phase and 4N fractions, we estimated the sensitivity and specificity of these fractions for detecting biopsies with *TP53* mutation and LOH (Fig. 3). Diploid S-phase fraction was a poor discriminator of biopsies with one or more *TP53* inactivation events (AUC, 0.61; 95% CI, 0.54-0.68), and 4N fraction was better but still not strong (AUC, 0.70; 95% CI, 0.63-0.77). 4N fraction was a good discriminator of biopsies with both *TP53* mutation and LOH (AUC, 0.92; 95% CI, 0.87-

0.97), but diploid S-phase fraction was not (AUC, 0.70; 95% CI, 0.60-0.79).

Discussion

Increased cellular proliferation has been reported to be associated with Barrett's esophagus neoplastic progression in cross-sectional studies that used a variety of methods to evaluate proliferative or S-phase fractions, including tritiated thymidine incorporation; monoclonal antibodies such as Ki67, proliferating cell nuclear antigen, cyclin A, and minichromosome maintenance proteins; and flow cytometry (17–26). However, to our knowledge, no previous studies have been conducted in which patients were characterized by an unbiased sampling protocol for total proliferative and cell cycle fractions and followed prospectively for the development of cancer as an end point. In this prospective cohort study (a phase 4 study, as defined by the Early Detection Research Network of the National Cancer Institute), we report that an elevated 4N fraction in biopsies from Barrett's esophagus is a strong and significant predictor of progression from Barrett's esophagus to esophageal adenocarcinoma (*P* < 0.0001), and diploid S-phase fractions had a smaller but significant (*P* = 0.03) association with the future development of esophageal adenocarcinoma. These results are consistent with an earlier analysis of our cohort, which found that a 4N fraction of 6% is the optimal threshold for defining elevated 4N for cancer risk prediction, indicating that 4N fraction should not be treated as a continuous value (23). However, total Ki67-positive proliferative fractions and G₁ fractions in diploid biopsies were not associated with progression to esophageal adenocarcinoma (*P* = 0.13 and *P* = 0.15, respectively). There was a low correlation coefficient between the Ki67-positive and S-phase fractions in diploid biopsies, indicating that progression to esophageal adenocarcinoma is more closely associated with

S phase (an increased G_1 to S phase transition) than with total proliferative fraction (G_0 to G_1 transition).

In cross-sectional studies, elevated cellular proliferation has been associated with risk factors of neoplastic progression in colonic epithelium, which, like metaplastic Barrett's epithelium, is formed of crypts. High S-phase fractions measured using [3 H]thymidine have been observed in normal-appearing mucosa in patients with adenomas (42), and high proliferating cell nuclear antigen-stained fractions in colorectal epithelial biopsies have been associated with risk factors for colorectal cancer (43). However, no association was found between

increased proliferative fractions based on proliferating cell nuclear antigen expression and future development of adenomas in a large prospective study of colon cancer patients (44). In another study, the unusual distribution of Ki67-stained cells in colonic crypts, but not the total fraction of Ki67-positive epithelial cells, was associated with an increased risk of development of secondary tumors in participants who had early colorectal tumors (45). Our study of Barrett's mucosal diploid biopsies agrees with these observations that overall cell proliferation has not been found to predict cancer outcome in the colon.

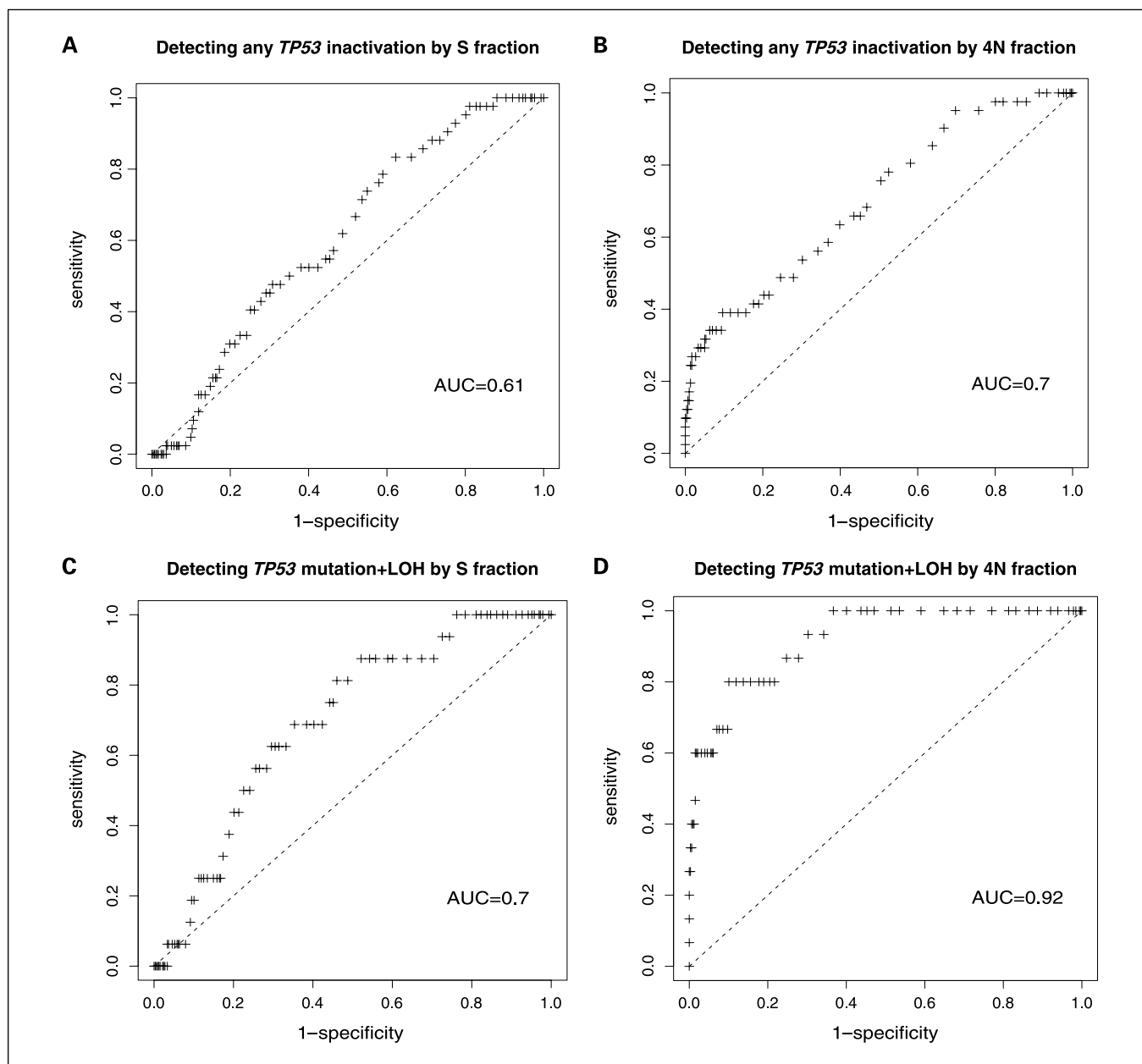


Fig. 3. Receiver operating characteristic curves for detecting *TP53* inactivation by mutation or LOH in diploid samples using S or 4N fractions. *A* and *B*, receiver operating characteristic (ROC) curves for discriminating between biopsies with no detectable *TP53* inactivation and those with any inactivation. Forty-two of 344 biopsies in which *TP53* inactivation and S-phase fractions were ascertained had detectable *TP53* mutation or LOH, and 41 of 342 biopsies in which *TP53* inactivation and 4N fractions were ascertained had detectable *TP53* mutation or LOH. The associated AUCs are indicated. *C* and *D*, receiver operating characteristic curves for discriminating between biopsies with both forms of *TP53* inactivation (mutation and LOH) and all other biopsies. Sixteen of 344 biopsies had detectable *TP53* mutation and LOH in the S-phase plot, and 15 of 342 biopsies had detectable *TP53* mutation and LOH in the 4N plot.

We also evaluated inactivation of the tumor suppressor genes *CDKN2A* and *TP53*, which develop in diploid cells before aneuploidy and esophageal adenocarcinoma, and the cell cycle to determine possible associations between proliferation and somatic genetic and epigenetic changes during neoplastic progression (35, 41, 46). *TP53* is involved in the G₁-S checkpoint (8); thus, inactivation of this tumor suppressor would be expected to increase the numbers of cells in S and G₂-M phases of the cell cycle. Here, we report that elevated diploid S and 4N fractions, but not diploid Ki67-positive or G₁ fractions, were associated with *TP53* inactivation in Barrett's esophagus biopsies. These results are consistent with the known negative regulatory functions of *TP53* in the G₁-S transition in model systems. *TP53* mutation and LOH were found in the same biopsies more frequently than expected by chance, suggesting that biallelic inactivation of *TP53* is selected, which is consistent with Knudson's two-hit hypothesis for inactivating tumor suppressor genes and previous studies of our cohort (41, 47). We found that high 4N fractions were a sensitive and specific indicator of biallelic *TP53* inactivation (Fig. 3). Elevated 4N fractions in a *TP53*-deficient background can consist of diploid G₂-M cells and tetraploid G₀ cells that develop from cells that do not complete chromosome segregation (48). Studies in rodents and humans have observed 4N populations in neoplasia (49). Further, studies have reported that tetraploid cell populations are genetically unstable intermediates that develop aneuploidy during neoplastic progression to cancer (10, 28, 46, 50–54). However, murine models seem to differ from humans in the stringency of mitotic checkpoints, and cycling tetraploids are observed more frequently in rodent models (46, 49–54). 4N populations in our study of Barrett's esophagus rarely have detectable tetraploid S-phase or G₂-M fractions by flow cytometry. *In vitro*, *TP53*-deficient Barrett's esophagus cell cultures express a number of transcripts associated with G₂-M and seem to be heterogeneous by fluorescence *in situ* hybridization analysis, suggesting that they may have an extremely prolonged G₂-M delay or the cells with genetic damage are arresting at a variety of G₂-M checkpoints (48). Because *TP53* abnormalities can be pleiotropic and could be selected for loss of proliferative control, decreased apoptosis, or gain of function, it will be difficult to determine the exact mechanism in human biopsies *in vivo*, given the heterogeneity in both constitutive and neoplastic genetic backgrounds and uncontrolled environment exposures. This may be a case in which a translational research study identifies an important biological problem that can then be addressed in model systems.

CDKN2A negatively regulates the transition from G₁ to S phase (7), so we had expected to observe higher S-phase and 4N fractions in samples with *CDKN2A* inactivation. However, because at least one allele of *CDKN2A* was inactivated in most of our samples, it was difficult to evaluate the effects of *CDKN2A* inactivation on proliferation and cell cycle in the absence of sufficient wild-type samples with Barrett's esophagus to use as a comparison group. *CDKN2A* methylation and LOH were detected in the same biopsies less frequently than expected by chance, which suggests that they are alternate pathways of *p16* inactivation or that the methylated alleles of *CDKN2A* can be lost. We did not find an association between inactivation of

CDKN2A or *TP53* and increased Ki67-positive or G₁ fractions, a result consistent with their known biological properties in studies in model systems.

Our study has several strengths: (a) the prospective cohort study design and long-term follow-up; (b) a defined, unbiased protocol for biopsy collection; (c) use of primary cancer rather than a surrogate such as incident high-grade dysplasia as an end point (30, 55, 56); (d) the measurement of total proliferation, cell cycle fractions, *CDKN2A* and *TP53* inactivation in the same cells; and (e) the use of flow cytometry instead of immunohistochemistry to obtain more precise estimates of Ki67-positive and cell cycle fractions based on an average of 15,000 cells per sample. However, immunohistochemistry can be used to determine the location of the proliferating cells within Barrett's epithelium, which may expand toward the luminal surface during neoplastic progression (22). There is also the concern that Ki67 labeling might not be specific for epithelial cells in Barrett's esophagus biopsies. However, we rarely observed nonepithelial Ki67-positive cells in Barrett's esophagus biopsy slides in earlier studies (57, 58). In addition, a previous study reported that an average of 90% of proliferating cells in whole endoscopic biopsies from Barrett's metaplasia were epithelial in origin (21). Finally, although proton pump inhibitor use has been associated with reduced proliferative fractions in Barrett's esophagus (59, 60), our study did not examine their effects because most patients in the cohort were managed with proton pump inhibitors and we did not have a comparison group. Proton pump inhibitor use can alleviate the symptoms of gastroesophageal reflux, but its effects on the development of cancer are not known, although dose can be studied in a randomized trial such as AspECT (61).

In a recent study of the same cohort, the best predictor of cancer outcome was a combination of chromosomal instability biomarkers that included early (9p LOH), intermediate (17p LOH), and late (tetraploidy and aneuploidy) events in neoplastic progression (33). Detection of *CDKN2A* and *TP53* loss is a promising biomarker for predicting neoplastic progression in many premalignant conditions because they are among the most common somatic genetic abnormalities in human cancers (14, 15). However, LOH at *TP53* and DNA content abnormalities (high 4N, aneuploidy) have independent contributions to cancer risk prediction (33), indicating that, despite the strong association between genetic (*TP53* inactivation) and cell cycle abnormalities (high 4N fractions) found in the present study, the characterization of both can improve cancer risk stratification in the esophagus and very likely other organs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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