

# Genetic Mechanisms of TP53 Loss of Heterozygosity in Barrett's Esophagus: Implications for Biomarker Validation

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## Abstract

**Background and Aims:** 17p (TP53) loss of heterozygosity (LOH) has been reported to be predictive of progression from Barrett's esophagus to esophageal adenocarcinoma, but the mechanism by which TP53 LOH develops is unknown. It could be (a) DNA deletion, (b) LOH without copy number change, or (c) tetraploidy followed by genetic loss. If an alternative biomarker assay, such as fluorescence *in situ* hybridization (FISH), provided equivalent results, then translation to the clinic might be accelerated, because LOH genotyping is presently limited to research centers.

**Methods:** We evaluated mechanisms of TP53 LOH to determine if FISH and TP53 LOH provided equivalent results on the same flow-sorted samples ( $n = 43$ ) representing established stages of clonal progression (diploid, diploid with TP53 LOH, aneuploid) in 19 esophagectomy specimens. **Results:** LOH developed by all three mechanisms: 32% had DNA deletions, 32% had no copy number change, and 37%

had FISH patterns consistent with a tetraploid intermediate followed by genetic loss. Thus, FISH and LOH are not equivalent ( $P < 0.000001$ ).

**Conclusions:** LOH develops by multiple chromosome mechanisms in Barrett's esophagus, all of which can be detected by genotyping. FISH cannot detect LOH without copy number change, and dual-probe FISH is required to detect the complex genetic changes associated with a tetraploid intermediate. Alternative biomarker assay development should be guided by appreciation and evaluation of the biological mechanisms generating the biomarker abnormality to detect potential sources of discordance. FISH will require validation in adequately powered longitudinal studies before implementation as a clinical diagnostic for esophageal adenocarcinoma risk prediction. (Cancer Epidemiol Biomarkers Prev 2006; 15(3):509–16)

## Introduction

The incidence of esophageal adenocarcinoma in the United States has increased dramatically since the 1970s, rising >400% in White males, the most commonly affected group (1). Increases have also been reported in White females and African Americans (1, 2). Barrett's esophagus, the only known precursor to esophageal adenocarcinoma, develops as a complication in ~5% to 10% of people with chronic gastroesophageal reflux disease. Although patients with Barrett's esophagus have an increased risk of developing esophageal adenocarcinoma compared with the general population, their absolute risk remains low (0.5-1.0% per year), and most will neither develop nor die of esophageal adenocarcinoma (3-8). Thus, periodic endoscopic biopsy surveillance has been recommended for early detection of cancer in patients with Barrett's esophagus (9, 10).

Progression of Barrett's esophagus to esophageal adenocarcinoma has been classically believed to follow a histologic pathway of negative for dysplasia, indefinite for dysplasia, low-grade dysplasia, high-grade dysplasia, and, finally, esophageal adenocarcinoma. Therefore, most surveillance

programs are based on periodic endoscopic biopsies evaluated for dysplasia, with patients with higher dysplasia grades being placed in more frequent surveillance (9, 10). However, present surveillance practices have come under increased scrutiny for several reasons, including observer variation in dysplasia classification (11-16), biological heterogeneity in dysplasia outcome (17-25), reduced adherence to standards of Barrett's care developed in specialty centers (26, 27), and questions of the success of early detection in standard endoscopic practice (8, 23, 25, 28).

Thus, increasing attention has been paid to the development of genetic and molecular biomarkers that can aid in identification of Barrett's esophagus patients who will and will not progress to esophageal adenocarcinoma (29-31). The National Cancer Institute's Early Detection Research Network has recently described five phases of biomarker development for early detection of cancer (32). Flow cytometric abnormalities were the first molecular biomarkers shown to identify subsets of patients at higher risk of progression from Barrett's esophagus to esophageal adenocarcinoma in prospective, Early Detection Research Network classification phase 4 studies (33-35). Patients with tetraploidy (4N fractions >6%), aneuploidy, or both in Barrett's esophagus have a markedly increased risk of developing esophageal adenocarcinoma compared with patients whose biopsies are diploid by flow cytometry (34, 35).

TP53, a tumor suppressor gene located on the short arm of chromosome 17 (17p), is often inactivated by a two-hit mechanism that involves mutation of one allele and loss of heterozygosity (LOH) of the other (36). Somatic genetic abnormalities involving TP53 are the most common genetic

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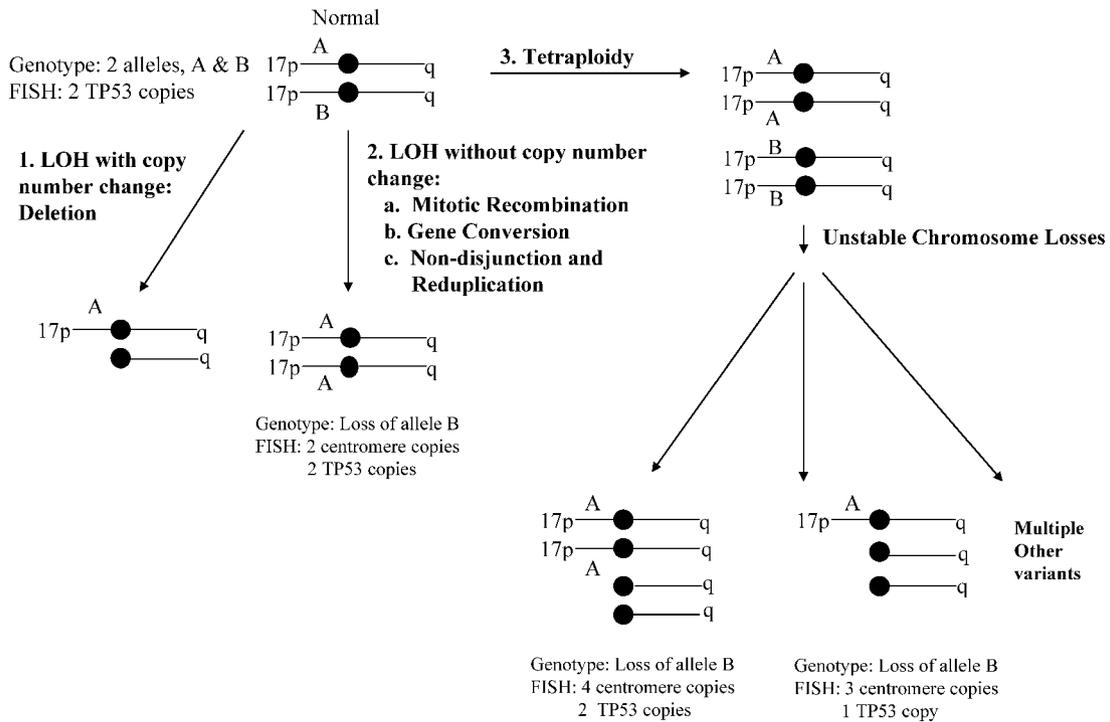
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lesions in human cancer and consistently develop before cancer. For example, 17p (TP53) LOH precedes development of high-grade dysplasia, tetraploidy, and aneuploidy in Barrett's esophagus (37-39). 17p (TP53) LOH has also been shown to be predictive of neoplastic progression in two prospective, Early Detection Research Network classification phase 4 studies of Barrett's esophagus, one using a cancer end point and the second using dysplasia as an end point (39, 40). In the prospective cohort study with a cancer end point, patients with 17p (TP53) LOH at baseline had a 16-fold increased risk of progression to esophageal adenocarcinoma compared with those without 17p (TP53) LOH (39).

Translation of the 17p (TP53) LOH results to the clinic would be facilitated if an alternative assay already in clinical use provided equivalent results. Fluorescence *in situ* hybridization (FISH) has the advantage of being potentially widely available in clinical laboratories, whereas high-throughput genotyping for LOH is currently limited to research centers. However, FISH and LOH measure two related, but potentially different, biomarkers. FISH measures physical DNA copy number change, whereas LOH measures genetic changes in loci. FISH and LOH might, therefore, provide equivalent information if all 17p (TP53) LOH events had copy number losses detectable by FISH. We hypothesized that if FISH provides equivalent results and has a high sensitivity and specificity for detection of 17p (TP53) LOH, then FISH might be considered as an alternative

biomarker assay to accelerate translation toward diagnostic application.

There are three general genetic mechanisms for developing LOH that have different implications for validation of FISH as a biomarker in Barrett's esophagus (Fig. 1). (a) LOH with copy number change: There can be copy number change of the TP53 locus (e.g., by deletions involving the 17p arm or nondisjunction of the entire chromosome 17; ref. 41). (b) LOH without copy number change: Mitotic recombination, gene conversion, or chromosome nondisjunction followed by reduplication could lead to LOH without copy number change as has been recently reported in breast cancer, bladder cancer, colonic adenomas, retinoblastoma, and acute myeloid leukemia (42-48). (c) LOH with complex copy number change: A more complex mechanism involving an unstable tetraploid intermediate with subsequent chromosome deletions and losses that generate genetic variants, leading to aneuploidy, and cancer has also been proposed (49). In prospective studies, we have reported previously that 17p (TP53) LOH is associated with an unstable tetraploid intermediate that precedes development of aneuploidy by ~17 months (37). Genetic assays that assess DNA polymorphisms detect LOH arising by all of these mechanisms, whereas other types of assays, including FISH and comparative genomic hybridization, detect only copy number changes. These chromosomal mechanisms can be tested by comparing 17p (TP53) LOH analysis and dual-probe FISH using one probe to the TP53 locus and another to the



**Figure 1.** Mechanisms of 17p (p53) LOH in Barrett's esophagus and implications for biomarker validation. Each individual has two normal copies of chromosome 17 and p53, which resides on the short arm of 17 (17p), one inherited from the father and the other from the mother. Circle, chromosome 17 centromere. The two p53 copies can be distinguished genetically as A and B. Mechanism 1: LOH with copy number change. Deletion of the portion of chromosome housing the p53 locus results in loss of allele B by genotyping and loss of one copy ("copy number change") of p53 by FISH, but two copies of the centromere remain. In this case, both genotyping and FISH would provide the same result because both show loss of one copy of p53. Mechanism 2: LOH without copy number change. Mitotic recombination, gene conversion, and nondisjunction of the entire chromosome with reduplication of the remaining chromosome all lead to loss of allele B, but there are two copies of both the centromere and the p53 locus. There would be discordance between genotyping (LOH) and FISH (two copies of p53) with any of these genetic mechanisms. Mechanism 3: Tetraploidy followed by genetic loss. The third mechanism generates a tetraploid intermediate with 4 centromeres and 4 p53 copies followed by random loss of chromosomes and arms, generating a large number of variants, all of which have lost allele B but which have 1 to 4 centromeres and 0 to 4 copies of the p53 locus. In this case, genotyping and FISH may give discordant results because genotyping can show LOH in cases where FISH shows 2 (or more) copies of p53. Simultaneous use of a chromosome 17 centromeric FISH probe may improve detection of the abnormalities in this case.

centromere (Fig. 1). If 17p (TP53) LOH occurs through copy number change with deletion of the TP53 locus, then only one TP53 locus would be detected by FISH. If 17p (TP53) LOH occurs as a result of chromosome mechanisms that do not result in copy number change, such as mitotic recombination, gene conversion, or chromosome nondisjunction and reduplication, then 2 TP53 loci would be detected by FISH. If a more complex mechanism involving an unstable tetraploid intermediate occurs, a greater diversity of FISH patterns would be predicted, including instances of 4 centromere markers, but fewer chromosome arm loci. Thus, if all TP53 LOH events arise by simple deletion (mechanism 1), then genotyping and FISH will yield concordant results, and FISH and LOH would be equivalent assays for the underlying biomarker. However, genotyping and FISH will always be discordant in cases of LOH that arise by mechanism 2 and there is a potential for discordance in cases arising by mechanism 3 (Fig. 1).

Although these mechanisms, especially deletion and mitotic recombination, have been elegantly studied in model organisms, such as the mouse (46, 50), investigations of the genetic mechanisms for LOH in human neoplastic progression are surprisingly rare, although the results have significant implications for biomarker development and validation. Although previous studies have reported some cases of discordance between LOH and FISH in several types of neoplasms, there have been several limitations noted in these studies. These limitations have included (a) small sample sizes, typically <10; (b) sampling limitations because different cell populations were assessed for FISH and LOH; (c) use of genetic loci for genotyping that did not correspond to the FISH probe; and (d) studies of a limited spectrum of disease (e.g., only small adenomas or cell lines and xenografts).

We evaluated mapped biopsies from esophagectomy specimens, where we have shown previously that esophageal adenocarcinomas arise in genetically abnormal fields with 17p (TP53) LOH, tetraploidy and aneuploidy (51). The same flow-sorted populations were assessed by FISH and LOH for each sample to eliminate sampling limitations. In addition, we compared 17p (TP53) LOH assessed by the same intragenic and flanking microsatellite polymorphisms used in the prospective, phase 4 study to copy number assessed by a FISH probe to the TP53 locus. Our study had three objectives. (a) The mechanisms of 17p (TP53) LOH were investigated by comparing FISH patterns in each patient to LOH results. (b) We tested the hypothesis that 17p (TP53) LOH and TP53 copy number change by FISH produce equivalent results to determine whether dual-probe FISH could be an alternative assay to high-throughput genotyping for LOH in Barrett's esophagus. (c) Single- and dual-probe FISH were compared.

## Materials and Methods

**Patient Selection and Tissue Acquisition.** Nineteen patients were selected for this study from the Seattle Barrett's Esophagus Study. All patients had undergone esophagectomy for high-grade dysplasia where intramucosal carcinoma could not be excluded (1 patient) or esophageal adenocarcinoma (18 patients). Biopsies were taken from the esophagectomy specimen in a grid like pattern as described previously to ensure collection of samples from a field surrounding the cancers, when available (52). Forty-three flow-sorted samples from 32 biopsies in these 19 esophagectomy specimens (mean, 2.3 per patient; range, 1-5) were taken remote from the adenocarcinoma ( $n = 25$  biopsies) and within the cancers ( $n = 7$  biopsies) depending on tissue availability. 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 institutional review board of the Fred Hutchinson Cancer

Research Center from 1993 to 2001. Since 2001, the study has been approved annually by the institutional review board of the Fred Hutchinson Cancer Research Center with reciprocity from the Human Subjects Division of the University of Washington.

**Ki-67/DNA Content Multivariable Flow Cytometric Sorting.** Each biopsy ( $n = 32$ ) was evaluated by flow cytometry, and diploid (G1), 4N, and aneuploid populations were separated, when present, and purified by flow sorting to separate proliferating and/or aneuploid epithelial cells from potentially contaminating genetically normal stromal cells (53-55). From these 32 biopsies, we obtained 43 flow cytometrically purified samples, including 30 diploid (24 from premalignant and 6 from within the cancer) and 13 aneuploid (8 from premalignant and 5 from within the cancer) fractions. Biopsies were processed for flow cytometry and analyzed by the computer program Multicycle (Phoenix Flow Systems, San Diego, CA) as described previously (55). At the time of sorting, nuclei were separated for FISH and genotyping from the same biopsy. Nuclei from each flow-purified sample were sorted directly onto a slide for FISH, and DNA was extracted from the remaining nuclei in the same flow-purified sample for genotyping so that FISH and LOH were evaluated on the same flow-purified population of cells.

**FISH Analysis.** Approximately 2,000 epithelial cells from each biopsy were sorted by flow cytometry, gating on the variables of diploid DNA content and Ki-67 positivity as described previously (54). Cells sorted onto plain glass slides in 5 mmol/L CaCl<sub>2</sub> were allowed to dry overnight and subsequently fixed using 3:1 methanol/acetic acid. FISH was done as described previously (56). In brief, FISH is done using the Vysis, Inc. (Downers Grove, IL) protocol. Fixed cells on slides were denatured in 78°C formamide, sent through the 70%, 85%, and 100% room temperature ethanol series, and then probed and incubated overnight for hybridization. The slides were then washed in 2× SSC and covered with Anti-fade (Oncor, Inc., Gaithersburg, MD) containing 0.25 ng/μL 4',6-diamidino-2-phenylindole (Accurate Chemical, Westbury, NY). Signals were scored at ×100 under oil using an epifluorescence microscope equipped with a Hamamatsu C5810 CCD camera. An average of 86 nuclei (range, 17-133) was counted per probe and sample pair. The number of nuclei counted was >50 in 35 of 43 (81%) samples. Probes used were TP53 (17p13.1) and CEP 17 (centromere) and directly conjugated as FITC/spectrum orange centromere/arm dual labels (Vysis). In this assay, the TP53 probe fluoresces red and the centromere fluoresces green.

The type and frequency of each FISH pattern was evaluated microscopically on an average of 86 cells. The modal FISH pattern in each sample was recorded for both dual- and single-probe FISH analysis. The presence of 2 centromeres and 2 TP53 probes was designated as FISH normal in dual-probe FISH analysis. The presence of 2 TP53 probes was designated as FISH normal by single-probe FISH analysis without regard to centromere status. All other patterns were designated as FISH abnormal. Dual-probe FISH done on control gastric biopsy samples revealed normal FISH patterns in 95% to 100% of the cells.

**17p (TP53) LOH Analysis.** 17p Genotypic analysis was done on 43 flow cytometrically purified DNA samples from 32 biopsies with 3 gastric controls evaluated per patient. DNA was extracted from the flow-purified neoplastic populations of each biopsy using either standard phenol/chloroform or the Puregene DNA Isolation kit recommended by the manufacturer (Gentra Systems, Minneapolis, MN). Whole genome amplification, genotyping, and LOH analyses with locus-specific polymorphisms D17S1537 (17p13.2), TP53 (17p13.1) pentanucleotide repeat, TP53 (17p13.1) dinucleotide, D17S786

(17p13.1), and D17S974 (17p12) were done using a normalized allele ratio (QLOH) cutoff of <0.4 at intragenic TP53 loci or loci spanning the TP53 locus as described previously (54). To prevent allelic drop-off, which can cause false-positive LOH at low input DNA levels, we used an average of 3,000 cell DNA equivalents for primer extension preamplification with a minimum DNA input that was 15-fold greater than that associated with false positivity (57). The presence or absence of LOH affecting the TP53 locus was then compared with the number of FISH spots for the TP53 locus on the same flow-sorted sample.

**Data Analysis.** The samples evaluated by FISH were compared against the genotyping results, which were used as the gold standard for LOH detection because it has been shown previously to predict progression to esophageal adenocarcinoma in a prospective, phase 4 biomarker study (39, 40). Sensitivity and specificity were calculated both for single- and dual-probe FISH. Two-tailed Fisher's exact test (58) was used to compare both the sensitivities [true positive / (true positive + false negative)] and the specificities [true negative / (true negative + false positive)] calculated based on the  $2 \times 2$  tables. To compare LOH detection by genotyping and FISH, the frequencies of detecting LOH generated by the three mechanisms (as shown in Fig. 1) for the two methods were arranged as  $2 \times 3$  table data. For the  $2 \times 3$  table data, simulated exact *P* value was used for the comparison (59).

## Results

A description of the study patients is provided in Table 1. These patients, who had progressed to cancer, were representative of the outcome of community surveillance programs where cancers are shifted to earlier stages (60-62). None received radiation or chemotherapy before tissue acquisition. The gender imbalance in the cases was not significantly different from the gender imbalance (4:1) in the population of Barrett's patients (*P* = 0.34).

Thirteen of 19 (68%) patients, including 19 of 43 DNA samples, had 17p (TP53) LOH by microsatellite genotyping, consistent with the known high association of 17p (TP53) LOH and the development of cancer in Barrett's esophagus (38, 39, 53, 63, 64). Of the 19 samples that had 17p (TP53) LOH by genotyping, 6 (32%) had a modal FISH pattern with <2 TP53 loci, consistent with a DNA copy number loss on chromosome arm 17p as the mechanism for LOH (Fig. 1; mechanism 1). Six (32%) samples with LOH by genotyping had a modal pattern of 2 centromeres and 2 TP53 loci, indicating LOH without DNA copy number change (Fig. 1; mechanism 2). We repeated genotyping with genomic DNA from 4 of these samples, and in each case, the LOH results were confirmed (data not shown). In all cases in which the modal FISH count at the TP53 locus was 2 or in which the modal pattern was 2 centromeres and 2 TP53 loci, this pattern was also the majority (>50%) population. Seven (37%) samples with LOH by genotyping had a modal FISH pattern containing 4 centromeres with

**Table 1. Patient characteristics**

Gender	
Male	18
Female	1
Mean age (range), y	64 (36-82)
Mean segment length (range), cm	8.4 (3-14)
Cancer depth of invasion	
High-grade dysplasia cannot rule out cancer	1
Intramucosal	10
Submucosal	3
Muscularis propria or deeper	5

**Table 2. Sensitivity of dual-probe FISH**

	LOH	No LOH	
FISH abnormal	13	1	Sensitivity, 68.4%
FISH normal	6	23	Specificity, 95.8%

multiple variants having different FISH patterns with fewer centromeres and arms, suggesting that a genetically unstable tetraploid intermediate is generating multiple abnormal subpopulations by chromosome losses and deletions, consistent with previous observations (Fig. 1; mechanism 3; refs. 37, 49). Thus, FISH and LOH detect different, but overlapping, genetic mechanisms that lead to loss of genetic information, and the hypothesis that the two assays can be considered equivalent can be rejected with a high degree of certainty (*P* < 0.000001).

From each patient, we evaluated premalignant samples distant from the tumors representing fields that cross-sectional genetic dependency and prospective studies have shown develop by clonal expansion and are clonal precursors of esophageal adenocarcinoma (38, 39, 65-69). We found that 17p (TP53) LOH developed by multiple genetic mechanisms in both diploid and aneuploid cell populations. Thus, 8 of 24 diploid, premalignant samples had 17p (TP53) LOH when assessed by genotyping; 5 of these 8 cases had a normal modal FISH pattern (2 centromeres, 2 TP53 loci), indicating that LOH developed because of genetic mechanisms, such as mitotic recombination, gene conversion, or chromosome nondisjunction followed by reduplication, which do not lead to copy number change. In the remaining three diploid, premalignant biopsies, an abnormal modal FISH pattern with TP53 copy number change (2 centromeres and 0/1 TP53 loci) was found. Biopsies with aneuploid cell populations generally had 17p (TP53) LOH [11 of 13 (85%) biopsies], consistent with the known association of these two lesions in Barrett's esophagus (38, 39, 70, 71). In 7 of 11 aneuploid samples with LOH by genotyping, the modal FISH pattern was consistent with a genetically unstable tetraploid intermediate with resulting deletions and chromosome losses (4 centromeres, 2 TP53 loci; 4 centromeres, 1 TP53 locus; or 3 centromeres, 2 TP53 loci). Three aneuploid samples had a modal pattern of 2 centromeres and 1 TP53 locus or 2 centromeres and 0 TP53 loci, consistent with deletion and copy number change. The remaining aneuploid sample with LOH had a modal normal FISH pattern with 2 centromeres and 2 TP53 loci, consistent with mitotic recombination, gene conversion, or nondisjunction and reduplication. The two flow cytometric aneuploid samples with no LOH by genotyping also had a modal FISH pattern of 2 centromeres and 2 TP53 loci (FISH normal). Thus, 17p (TP53) LOH can be detected in both diploid and aneuploid cell populations by genetic mechanisms with and without copy number change (Fig. 1).

Overall, dual-probe FISH was only moderately sensitive although very specific as an alternative biomarker assay for detection of LOH in this study (Table 2). It is of note, however, that there was a trend to lower FISH sensitivity in the diploid samples representing an early stage of clonal evolution: in this subset, dual-probe FISH was abnormal in only 3 of the 8 (38%) LOH events, although the patients clearly progressed to cancer. In the 8 premalignant aneuploid samples, dual-probe FISH was abnormal in all 6 that had LOH. In the cancer aneuploid samples, 5 of 6 had LOH by genotyping, and dual-probe FISH was detected 4 of the 5, with 1 false negative. The single abnormal FISH result with no LOH occurred in a mixed-cancer diploid sample with a 49% modal FISH pattern of 2 centromeres and 1 TP53 locus and a normal FISH pattern of 44% with an intermediate genotyping call slightly outside the cutoff for LOH. In total, there was agreement between dual-probe FISH and genotyping in 36 of 43 samples, with a

sensitivity of 68% (95% confidence interval, 44-86%) and a specificity of 96% (95% confidence interval, 76.9-99.8%) for dual-probe FISH compared with genotyping as the gold standard for LOH detection. Dual-probe FISH had a positive predictive value of 93% and a negative predictive value of 79% for 17p (TP53) LOH detected by genotyping.

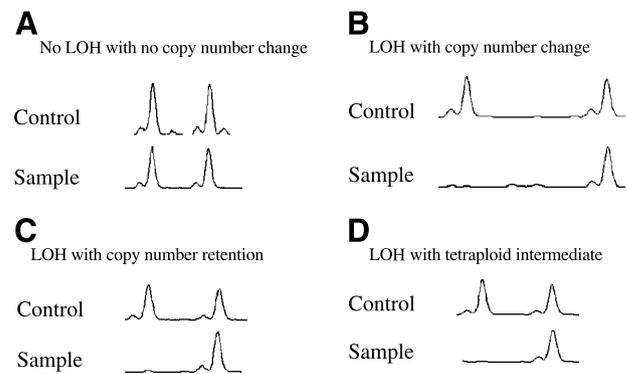
Single-probe FISH was markedly less sensitive than dual-probe FISH in detecting 17p (TP53) LOH (Table 3). In the premalignant, diploid group, single-probe FISH detected only 3 of 8 cases of LOH. In the premalignant, aneuploid samples, single-probe FISH detected only 1 of the 8 cases of LOH because some cases arose by mechanisms that did not lead to copy number changes and others arose via a tetraploid intermediate that could not be detected without the centromeric probe. Finally, single-probe FISH detected only 3 of 5 cases of LOH in the cancer aneuploid samples, with 1 false positive and 1 false negative. Overall, single-probe FISH had a sensitivity of 37% (95% confidence interval, 17.2-61.4%) and a specificity of 96% (95% confidence interval, 76.9-99.8%). The sensitivity of dual-probe FISH is significantly higher than that of single-probe FISH ( $P < 0.03$ , Fisher's exact test), whereas the specificities of the two methods are the same (Figs. 2 and 3).

## Discussion

Research from multiple laboratories has established that 17p LOH is (a) disease specific, found rarely, if ever, in normal tissues; (b) among the most common genetic abnormalities in human neoplasia and in esophageal adenocarcinoma; and (c) useful in identifying patients at high risk for cancer in Barrett's esophagus and other conditions (38-40, 53, 63, 64, 72). 17p (TP53) LOH identifies Barrett's esophagus patients with a 16-fold increased risk of progression to esophageal adenocarcinoma (39). To our knowledge, this is the first study to investigate genetic mechanisms of 17p (TP53) LOH in Barrett's esophagus, and the results have implications for biomarker validation in general. Our data indicate that FISH and LOH detect different but overlapping mechanisms that lead to loss of genetic information, and the hypothesis that they are equivalent can be rejected ( $P < 0.000001$ ).

The sensitivity of dual-probe FISH for 17p (TP53) LOH was greater than single-probe FISH ( $P < 0.03$ ). In this study, FISH had a lower detection rate of 17p (TP53) LOH early in progression (diploid samples) compared with late in progression (aneuploid samples;  $P < 0.04$ , two-tailed Fisher's exact test). The frequency of diploid samples with 17p (TP53) LOH in this study (42%) is not significantly different from what has been reported in a surveillance cohort (32%;  $P > 0.4$ ; ref. 39). If 17p (TP53) LOH typically develops at early stages of neoplastic progression by mechanisms that do not result in copy number change, then FISH and other assays that detect copy number changes may have an unacceptable false-negative rate for LOH detection.

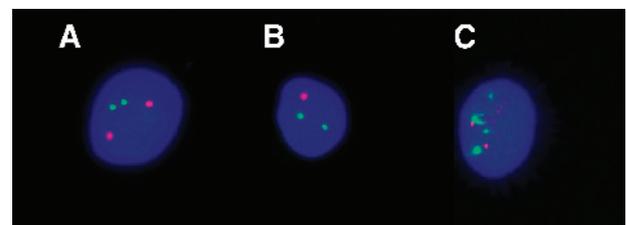
Comparison of allelotype and comparative genomic hybridization studies suggest that our results are representative of Barrett's esophagus and esophageal adenocarcinoma in general. 17p LOH has been found at high frequency in esophageal adenocarcinoma (63%, 75%, 96%, and 100%) in four studies (38, 63, 64, 73). In contrast, 17p copy number loss was detected in much lower frequencies by comparative genomic hybrid-



**Figure 2.** 17p (p53) LOH genotyping data. In each set, two p53 alleles are seen in the normal control above. The samples below reveal only one p53 allele, indicating LOH at the TP53 locus. The mechanism of LOH for each set is listed. **A.** No LOH, FISH normal. **B.** LOH, FISH copy number change. **C.** LOH, FISH no copy number change. **D.** LOH, FISH tetraploid intermediate.

ization (0%, 0%, 22%, 30%, 40%, and 47%) in six studies (74-79). Thus, our results and those of others indicate that some cases of LOH, including those that progress to cancer, will not be detected by copy number loss.

Surprisingly few studies have directly compared LOH to copy number change, and the number of cases in each study has been small. To our knowledge, the classic studies of retinoblastoma were the first to show conclusively that LOH could arise by a variety of genetic mechanisms with and without copy number change (42). Recent studies have reported that some cases of LOH do not have copy number changes detected by FISH, cytogenetics, or comparative genomic hybridization (41, 44, 48). A recent study of the Rb and TP53 loci using FISH and LOH reported 4 of 50 primary breast cancers in which LOH was not associated with copy number change (mechanism 2) and 1 case that was tetraploid for chromosome 17 (mechanism 3; ref. 43). Two studies of bladder cancer cell lines each reported 2 cases of LOH without copy number change (mechanism 2; refs. 44, 48). Thiagalingam et al. reported that 9 of 9 colon cancer xenografts or cell lines with LOH involving chromosomes 5, 8, 17, and 18 had evidence of dsDNA breaks and a small number cases with LOH had as many as four copies of chromosome 17 or 18 (mechanism 3; ref. 41). Although mechanisms of LOH have not been systematically studied in premalignant conditions, two studies have reported that 5q LOH in colorectal adenomas can develop by mitotic recombination (45, 46).



**Figure 3.** Representative dual-probe FISH patterns. The centromere is stained green and the p53 locus is stained red. **A.** normal cells have 2 chromosome 17 centromeres and 2 p53 loci (2 green, 2 red). **B.** nucleus with 17p (p53) LOH with deletion of p53 locus (copy number change). There are 2 green centromeres and 1 red p53 locus. **C.** this nucleus has 4 green centromeres and 2 red p53 loci, consistent with deletion of 17p from an unstable tetraploid intermediate.

**Table 3. Sensitivity of single-probe FISH**

	LOH	No LOH	
FISH abnormal	7	1	Sensitivity, 36.8%
FISH normal	12	23	Specificity, 95.8%

By evaluating the same flow-sorted populations for FISH and LOH using probes and microsatellite polymorphisms corresponding to the TP53 locus from samples representing stages of evolution of 17p (TP53) LOH (diploid, aneuploid, cancer) during neoplastic progression, our study has extended the results of the earlier investigations. Our use of the same cell populations for FISH and LOH analysis, while overcoming previous sampling limitations, resulted in some populations having relatively few cells for FISH analysis because flow-sorted cells are more dilute than cells *in situ*. However, we do not believe this affected our conclusions because only 8 (19%) samples had <50 nuclei evaluated by FISH and 7 of those had concordant LOH and FISH results. In the one discrepant case, LOH was detected by genotyping, but FISH showed 34 of 40 nuclei with 2 centromeres and 2 arms. Because at least 50% of cells in the population must have LOH for it to be detected, it is statistically highly unlikely that the FISH result could actually have been aneuploid ( $P = 0.0016$ ). Exclusion of the one discrepant sample did not affect our conclusions. Because our study design involved multiple samples from the same patient, there might be concern that a large clone with one abnormality might lead to overrepresentation of different patterns of LOH (e.g., LOH with normal copy number), but this does not seem to be the case. These samples came from 4 patients, and 2 patients each had two samples. Thus, the findings were identical in multiple patients and in duplicate samples of the same clone in single patients.

LOH cannot be detected reliably in populations in which less than half of the cells have LOH (54). All samples with normal modal FISH patterns had a modal population of >50%. We attempted to improve FISH sensitivity by combining all abnormal FISH subpopulations in these samples and decreasing the threshold percentage of cells that would result in a FISH abnormal call. This resulted in a modest improvement in sensitivity at a cost of progressively decreasing specificity. At a threshold of 20% abnormal FISH, the sensitivity for LOH was 78%, but the specificity dropped to 63%. It might be hypothesized that because FISH can detect abnormalities in a few cells or even a single cell, it may be more sensitive than LOH. However, sensitivity for a predictive biomarker is defined by its ability to predict future progression to cancer (32), and the available evidence indicates that the size of a 17p (TP53) LOH clone predicts cancer risk; larger clones progress more rapidly than small clones (66). Further, the fundamental result of our study and those of others is that some cases of 17p (TP53) LOH arise by genetic mechanisms that do not produce copy number changes, including cases that progress to cancer. Thus, FISH or other copy number assays cannot be equated to LOH, and adequately powered longitudinal (phase 3 and/or 4) studies will be required before considering FISH for clinical assessment of esophageal adenocarcinoma risk. In such studies, TP53 FISH or a combination of TP53 FISH and 17p (TP53) LOH may provide better, worse, or the same predictive information as 17p (TP53) LOH alone.

These results indicate that validation of biomarker assays should be guided by an appreciation of the biological and genetic mechanisms generating the biomarker abnormality. This principle may be useful in considering other biomarkers as well. For example, TP53 protein overexpression assessed by immunohistochemistry is frequently used as an alternative biomarker assay for TP53 mutation without consideration of potential false-positive or false-negative results. However, nonsense or frameshift mutations can lead to false-negative results because the protein is not expressed, and Barrett's esophagus and esophageal cancers have a relatively high frequency of false-negative and false-positive results for TP53 immunostaining compared with the gold standard of mutation detection (67, 68, 80-83). This potential for false-negative and false-positive results relative to the underlying biomarker, TP53 mutations, may contribute to variable success in cancer

risk prediction in case-control and prospective studies of Barrett's esophagus (84, 85).

Doak et al. have recently reported results of FISH on cytology brushings of patients with Barrett's esophagus, including probes for the p16 locus on chromosome 9p and the TP53 locus on chromosome 17p (86). Although they did not evaluate LOH by genotyping, they did report low frequencies of p16 and TP53 FISH abnormalities compared with other studies that have used genotyping (71, 80, 87, 88). This suggests that our results may be generalizable to patients in endoscopic surveillance and that many LOH events in Barrett's esophagus arise by mechanisms that do not produce copy number changes. Fahmy et al. reported results of FISH probes for the TP53 locus on 17p as well as other chromosomal loci in Barrett's endoscopic cytology samples (89). Although they also did not evaluate LOH, they reported that TP53 locus loss was associated with a gain of chromosome 17 centromeric copies in 50% of cases, consistent with our observation that this was a common finding in patients with 17p (TP53) LOH (mechanism 3). These results are consistent with a previous longitudinal study in Barrett's esophagus in which 17p (TP53) LOH was associated with development of a tetraploid population that rapidly progressed to aneuploidy (37).

The issue of alternative biomarker assays is likely to become increasingly common in biomarker validation. Rapid advances in discovery science are producing an increasing number of potential biomarker assays for clinical application, some of which, such as copy number change, might be considered as alternatives for existing biomarkers, such as LOH. Our results indicate that alternative assays should be directly compared with existing biomarker(s) early in phase 1 (discovery) and phase 2 (established disease) validation studies to detect suspected or unsuspected biological mechanisms that may result in discordant results. Such early comparisons are likely to facilitate biomarker selection and power calculations for more advanced validation studies, including clinical platform development and phase 3 (retrospective, longitudinal) and phase 4 (prospective) trials.

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## Genetic Mechanisms of TP53 Loss of Heterozygosity in Barrett's Esophagus: Implications for Biomarker Validation

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