



Original contribution

A comparison of conventional cytology, DNA ploidy analysis, and fluorescence in situ hybridization for the detection of dysplasia and adenocarcinoma in patients with Barrett's esophagus^{☆,☆☆}

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Received 28 December 2007; revised 1 February 2008; accepted 6 February 2008

Keywords:

Fluorescence in situ hybridization (FISH);
Cytology;
Barrett's esophagus;
Dysplasia;
Esophagus

Summary New detection methods with prognostic power are needed for early identification of dysplasia and esophageal adenocarcinoma (EA) in patients with Barrett's esophagus (BE). This study assessed the relative sensitivity and specificity of conventional cytology, DNA ploidy analysis with digital image analysis (DIA), and fluorescence in situ hybridization (FISH) for the detection of dysplasia and adenocarcinoma in endoscopic brushing specimens from 92 patients undergoing endoscopic surveillance for BE. FISH used probes to 8q24 (*C-MYC*), 9p21 (*P16*), 17q12 (*HER2*), and 20q13. Four-quadrant biopsies taken every centimeter throughout visible Barrett's mucosa were used as the gold standard. The sensitivity of cytology, DIA, and FISH for low-grade dysplasia was 5%, 5%, and 50%, respectively; for high-grade dysplasia (HGD), 32%, 45%, and 82%, respectively; and for EA, 45%, 45%, and 100%, respectively. FISH was more sensitive ($P < .05$) than cytology and DIA for low-grade dysplasia, HGD, and EA. The specificity of cytology, DIA, and FISH among patients ($n = 14$) with tissue showing only benign squamous mucosa was 93%, 86%, and 100% ($P = .22$), respectively. All patients with a polysomic FISH result had HGD and/or EA within 6 months ($n = 33$). There was a significant difference between FISH categories (negative, 9p21 loss, gain of a single locus, and polysomy) for progression to HGD/EA

[☆] This study was supported by Abbott Molecular (Des Plaines, IL), NIH grants R01CA09704 and CA85992-01, and the Mayo Foundation (Rochester, MN).

^{☆☆} It should be noted that a patent has been filed for the FISH probe set discussed in this study. Dr Halling and Ms Shannon Brankley are listed as inventors on the filed patent. Drs Halling, Kipp, and Wang, and Ms Shannon Brankley have the potential to receive royalties from the sale of this FISH probe set. This study was partially funded by Abbott Molecular, Inc. Dr Larry Morrison and Ms Mona Legator are Abbott Molecular employees. No other authors of this study have potential conflicts of interest regarding any of the products used in this study.

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($P < .001$). These findings suggest that FISH has high sensitivity for the detection of dysplasia and EA in BE patients, with the power to stratify patients by FISH abnormality for progression to HGD/EA. Additional studies are needed to further evaluate the clinical use of FISH.

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1. Introduction

Barrett's esophagus (BE) is a preneoplastic condition in which the squamous epithelium of the distal esophagus undergoes transformation to intestinal metaplasia (IM). It is estimated to affect 10% to 12% of patients with chronic gastroesophageal reflux disease [1], the most significant known risk factor in the development of BE, as well as 6% of those without symptoms [2]. Patients with BE are approximately 125 times more likely to develop carcinoma than the general population [3]. The evolution to cancer from Barrett's epithelium is generally accepted to be a multistep progression as follows: (1) IM without dysplasia, (2) low-grade dysplasia (LGD), (3) high-grade dysplasia (HGD), and (4) esophageal adenocarcinoma (EA).

The incidence of EA is increasing more rapidly than any other malignancy in the United States [4]. Because EA has a poor prognosis unless detected and treated at its earliest stages, the guidelines from gastrointestinal organizations have recommended that patients with BE undergo periodic endoscopic surveillance with biopsies [5]. However, the histologic diagnosis of dysplasia has several shortcomings, which include limited sampling of affected mucosa, extensive time required to obtain the recommended number of biopsies leading to lack of compliance, and relatively poor pathologic interobserver reproducibility [6-8].

Numerous studies suggest that there is an increase in the number of genetic alterations as IM progresses to EA [9-13]. Premalignant lesions such as LGD have been shown to be diploid or near diploid with frequent alterations in the *P16* and *P53* genes [12-16]. Alterations of these tumor suppressor genes are thought to promote tumorigenesis because of compromised cell cycle regulation, leading to abnormal cellular proliferation and increased genetic instability. The accumulation of genetic errors during progression to malignancy results in aneuploid lesions such as HGD and EA with hyperdiploid DNA content [12]. Genes or genetic loci found to be frequently altered in BE include 3p21, 5p15, 5q21-22, 7p12 (*EGFR*), 7q36.1, 8q24.12-13 (*C-MYC*), 9p21 (*P16*), 17p13.1 (*P53*), 17q12 (*HER2*), 20q13.2, and the Y chromosome [9,14,17]. A fluorescence in situ hybridization (FISH) probe set used to detect dysplasia and EA has been identified, which contains probes directed to loci 8q24 (*C-MYC*), 9p21 (*P16*), 17q12 (*HER2*), and 20q13 [13].

Techniques that assess for chromosomal alterations such as DNA ploidy analysis by flow cytometry or digital image analysis (DIA), loss of heterozygosity analysis, and FISH

can potentially be used to identify dysplasia and EA in patients with BE. Our study focuses on ancillary techniques that can be applied to cytology brushing specimens collected during endoscopy. Thus, the goal of this study was to assess the relative sensitivity and specificity of conventional cytology, DIA, and FISH for the detection of dysplasia and EA in patients with BE.

2. Materials and methods

2.1. Patients

This study used endoscopic brushing specimens from 97 patients undergoing surveillance for BE. Five specimens were excluded because of an inadequate number of cells for DIA and/or cytologic examination. The remaining 92 specimens were obtained from 83 males and 9 females with a mean age of 64.4 years (range, 34-87 years). Eighty-four patients had a history of biopsy-confirmed HGD and/or EA, 7 had a history of LGD, and 1 had a history of IM only.

2.2. Endoscopic brushing

Cytology specimens were obtained during endoscopy by sweeping 2 gastrointestinal brushes (Hobbs Medical Inc, Stafford Springs, CT) over the suspected area of IM, BE-associated neoplasia, or previously diagnosed BE. Each brush was immediately placed in a separate vial containing PreservCyt Solution (Cytoc Corporation, Boxborough, MA). One vial was used for the FISH assay, and the other was split equally for routine cytology and DIA processing.

2.3. Biopsy specimen

All patients had systematic biopsies performed at the time of endoscopy. These biopsies were taken using standard biopsy forceps in 4 quadrants every centimeter throughout the mucosa. Any visible abnormality suspicious for neoplasm was removed using endoscopic mucosal resection techniques and carefully sectioned for histology. Each tissue specimen was interpreted as part of routine clinical practice. The most advanced degree of histologic abnormality was used as the gold standard for determining the sensitivity and specificity of cytology, DIA, and FISH. All tissue diagnoses of HGD and EA were agreed upon by 2 gastrointestinal pathologists.

2.4. Routine cytology

Cytology slides were prepared with the ThinPrep method (Cytec Corporation) and Papanicolaou stained. Each specimen was evaluated by an experienced cytopathologist (T. J. Sebo) without knowledge of concordant biopsy results or patient history, and interpreted as negative, atypical, suspicious, or positive for cancer. Criteria for these categories were derived from a study by Geisinger et al [18], with the exception that our study considered reactive atypia as a separate category (“atypical”).

2.5. DIA for the determination of DNA ploidy

DNA ploidy analysis was performed as previously described using DIA [19]. Slides were prepared with the ThinPrep method and stained with the Feulgen dye. Fifty of the most abnormal-appearing cells were collected with a CAS 200 image analyzer (Bacus Laboratories, Lombard, IL), which measures the DNA content of cells and generates a DNA ploidy histogram. Results were categorized as diploid (DNA index = 0.95-1.10), aneuploid (DNA index = 1.11-1.89, >2.10), or tetraploid (DNA

index = 1.90-2.10) (Fig. 1). Aneuploid and tetraploid results were considered positive for malignancy.

2.6. Fluorescence in situ hybridization

FISH was performed as previously described using a probe set that was developed specifically for the detection of dysplasia and EA in patients with BE [13]. The pepsin (2500-3500 U/mg; Sigma-Aldrich, St Louis, MO) concentration used for prehybridization treatment was 0.05 mg/mL. The probe set consists of directly labeled DNA probes to 8q24 (*C-MYC*), 9p21 (*P16*), 17q12 (*HER2*), and 20q13. The number of signals for each of the 4 probes was enumerated in 100 consecutive noninflammatory nonsquamous cells. After the 100-cell enumeration, the remainder of the specimen was scanned for the most morphologically abnormal-appearing cells, and those demonstrating polysomic signal patterns were recorded.

A variety of chromosomal abnormalities were observed, including homozygous and hemizygous 9p21 loss, polysomy (3 or more copies for 2 or more of the 4 loci), tetrasomy (4 copies for each of the 4 loci), and gain of a single locus (Fig. 2). The cutoff values for these abnormalities (ie, the number of cells with a specific signal pattern required to reach positivity) that provided optimal sensitivity and specificity were determined using receiver operator characteristic curves on a separate cohort of high-risk patients (unpublished data). Based on those analyses, a specimen in this study was considered FISH positive if (1) 6% of the cells or higher showed homozygous 9p21 loss, (2) 11% of the cells or higher showed any combination of homozygous and hemizygous 9p21 loss, (3) 4% of the cells or higher exhibited polysomy, (4) 5% of the cells or higher exhibited tetrasomy, or (5) 5% of the cells or higher showed gain of a single locus. The specimen was also considered positive if scanning the remainder of the specimen after the 100-cell enumeration revealed 4 or more polysomic cells, or if 4 or more polysomic cells were present additively between the 100-cell count and the scan.

2.7. Follow-up data

Histologic results were collected for each patient by electronic chart review of all endoscopic visits after the FISH result. The most advanced level of histologic abnormality was recorded for each follow-up visit. A mean of 267 days of follow-up for patients in this study was collected (range, 0-1304 days).

2.8. Statistical analysis

Statistical analyses were performed with SPSS 11.5 software (SPSS, Chicago, IL). The McNemar and Cochran *Q* tests were used to compare the sensitivity and specificity of cytology, DIA, and FISH, as appropriate. Kaplan-Meier curves were generated using JMP 7.0 (SAS Institute,

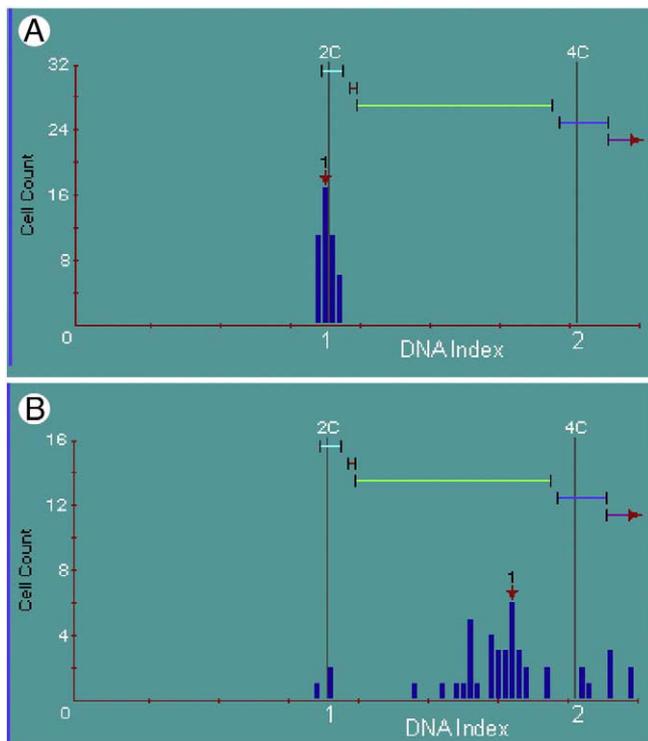


Fig. 1 Example of (A) diploid (negative) and (B) aneuploid (positive) DIA histograms. DNA content (x-axis) is plotted against the number of cells (y-axis). The DNA content of diploid cells is within the 2c range (DNA index = 0.95-1.10); tetraploid cells, within the 4c range (DNA index = 1.90-2.10); and aneuploid cells, between 2c and 4c or >4c (DNA index = 1.11-1.89; >2.10).

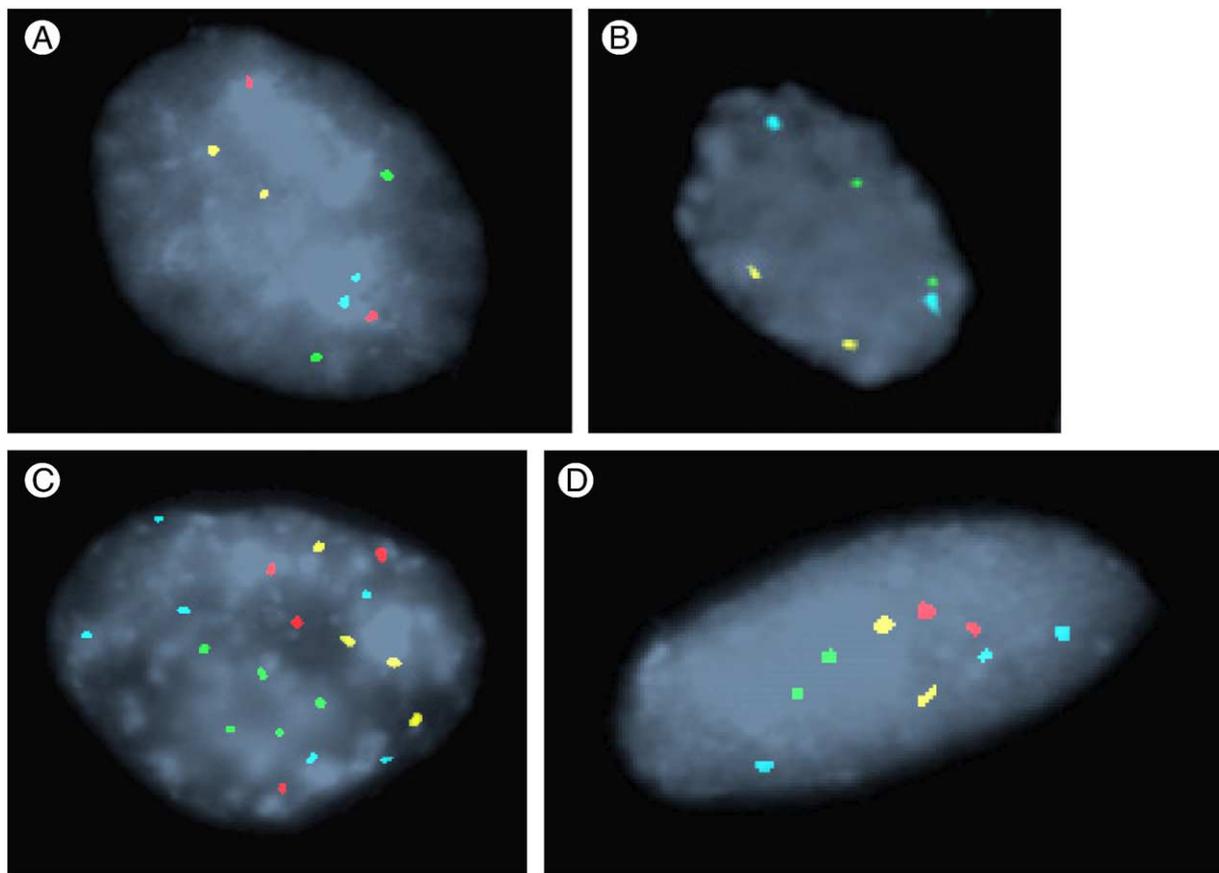


Fig. 2 Representative examples of FISH signal patterns: (A) normal (2 signals of each of the 4 probes), (B) homozygous 9p21 loss (no red signals), (C) polysomy (2 or more signals of 2 or more probes), (D) gain of 8q24 (>2 aqua signals). 9p21 (red), 17q12 (green), 8q24 (aqua), and 20q (gold).

Cary, NC). *P* values of .05 or lower were considered statistically significant.

3. Results

Ninety-two specimens had corresponding results for routine cytology, DIA, and FISH. The histologic findings for these patients were 14 with benign squamous mucosa, 16 with IM, 20 with LGD, 22 with HGD, and 20 with EA. The sensitivity and specificity of conventional cytology were determined using 3 different definitions of a positive diagnosis (Fig. 3). The most inclusive definition (“Cytology P + S + A”) considered positive, suspicious, and atypical cytologic diagnoses as a positive test result. “Cytology P + S” included positive and suspicious diagnoses, whereas “Cytology P” included only a positive diagnosis as a positive test result. As expected, “Cytology P + S + A” had the highest sensitivity because it included more specimens as positive for dysplasia/EA, but this was accompanied by a decrease in specificity.

“Cytology P + S” (referred to as cytology from this point forward) was used for comparison because this reflects how these interpretations would likely be used in clinical practice.

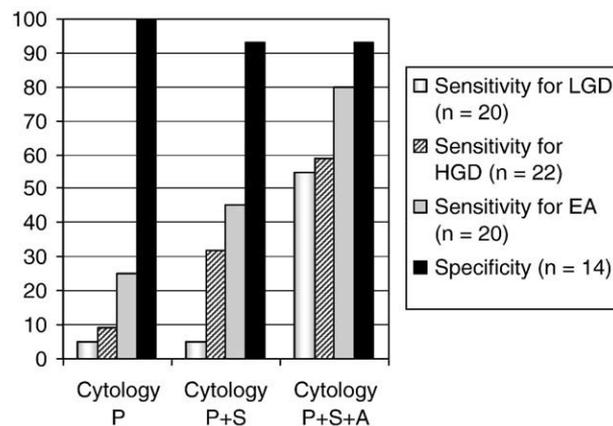


Fig. 3 Sensitivity and specificity of cytology using different interpretation cutoffs. “Cytology P”: only positive interpretations considered positive for dysplasia or EA; “Cytology P + S”: positive and suspicious interpretations considered positive for dysplasia or EA; “Cytology P + S + A”: positive, suspicious, and atypical cytologic interpretations considered positive for dysplasia or EA. Specificity is calculated by considering patients with benign squamous mucosa.

The sensitivity of cytology, DIA, and FISH for the detection of LGD, HGD, and EA is shown in Fig. 4A, and the specificity when considering patients with benign squamous mucosa is shown in Fig. 4B.

The sensitivity of cytology, DIA, and FISH for the detection of LGD was 5%, 5%, and 50%, respectively. FISH was significantly more sensitive than cytology ($P = .004$) and DIA ($P = .004$), but there was no difference between the sensitivity of cytology and DIA ($P = 1.000$) for LGD. The sensitivity of cytology, DIA, and FISH for the detection of HGD was 32%, 45%, and 82%, respectively. FISH was significantly more sensitive than cytology ($P = .002$) and DIA ($P = .016$), but there was no difference in the sensitivity of cytology and DIA ($P = .38$) for HGD. The sensitivity of cytology, DIA, and FISH for the detection of EA was 45%, 45%, and 100%, respectively. FISH was significantly more sensitive than cytology and DIA ($P = .001$) for the detection of EA. There was not a statistically significant difference in the sensitivity of cytology and DIA for EA ($P = 1.000$).

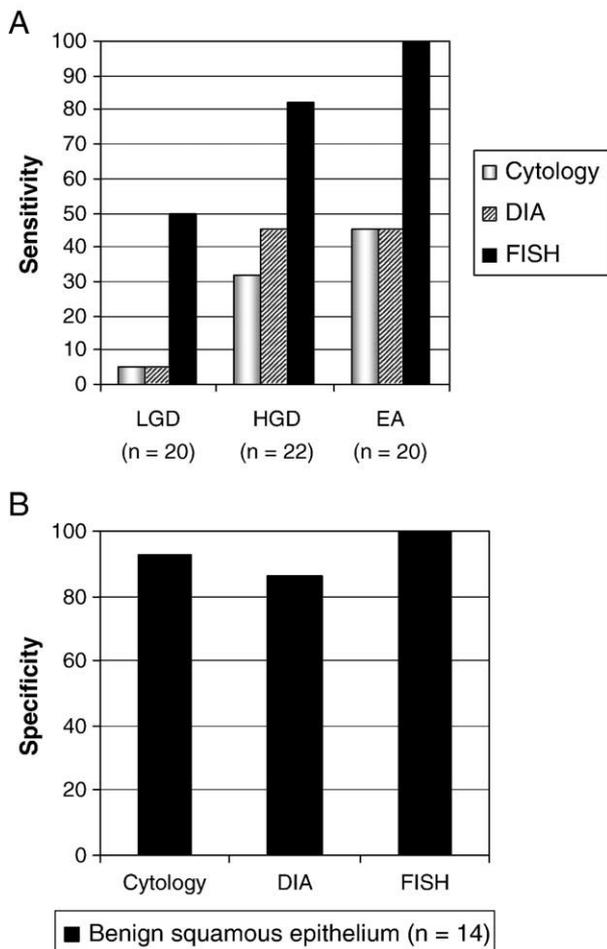


Fig. 4 A, Sensitivity of cytology, DIA, and FISH for the detection of LGD, HGD, and EA (cytology includes positive and suspicious interpretations). B, Specificity of cytology, DIA, and FISH when considering patients with benign squamous mucosa.

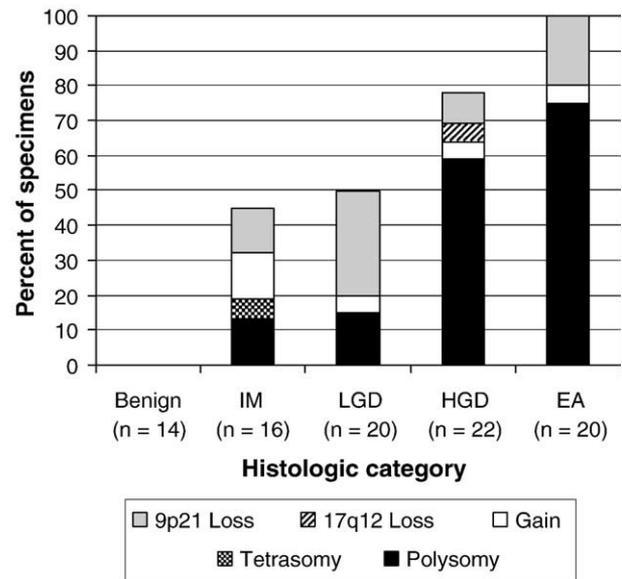


Fig. 5 The types of chromosomal abnormalities observed in brushing specimens among the FISH positive cases based upon the histologic result from the concurrent biopsy.

The specificity of cytology, DIA, and FISH among patients with a biopsy showing only benign squamous mucosa ($n = 14$) at the time of the brushing specimen was 93%, 86%, and 100%, respectively, which are not statistically different ($P = .22$).

The types of FISH abnormalities observed among brushing specimens diagnosed as positive by FISH for each histologic category are shown in Fig. 5. In patients with IM, 7 (44%) of 16 had positive FISH results displaying abnormalities that included 9p21 loss ($n = 2$), gain of 9p21 ($n = 1$), gain of 8q24 ($n = 1$), tetrasomy ($n = 1$), and polysomy ($n = 1$). In patients with LGD, 10 (50%) of 20 had positive FISH results displaying abnormalities that included 9p21 loss ($n = 6$), gain of 17q12 ($n = 1$), and polysomy ($n = 3$). In patients with HGD, 17 (77%) of 22 had positive FISH results displaying abnormalities that included 9p21 loss ($n = 2$), loss of both 17q12 and 9p21 ($n = 1$), gain of 8q24 ($n = 1$), and polysomy ($n = 13$). All 20 (100%) patients with EA had positive FISH results displaying abnormalities that included 9p21 loss ($n = 4$), gain of 8q24 ($n = 1$), and polysomy ($n = 17$).

To determine if FISH results were able to predict progression to HGD/EA over time, a Kaplan-Meier analysis was performed (Fig. 6). There was a significant difference over time between FISH diagnostic categories for progression to HGD/EA ($P < .001$).

4. Discussion

Successful surveillance of patients with BE for the detection of dysplasia and EA using a 4-quadrant biopsy for each 1 to 2 cm of affected mucosa is hindered by various

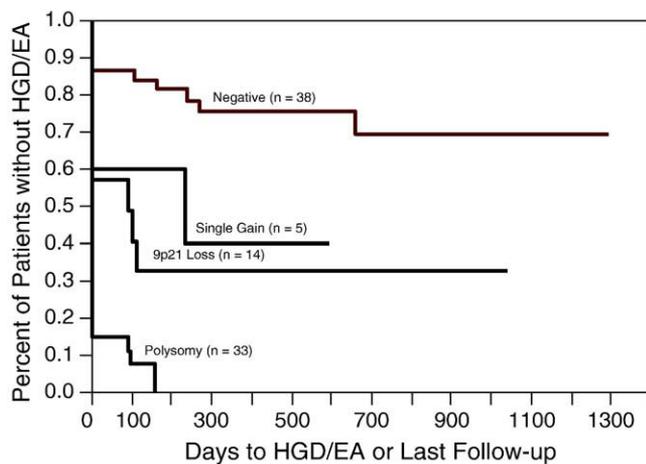


Fig. 6 Kaplan-Meier analysis demonstrates differences in time to HGD and/or EA for patients based upon the FISH abnormality found in initial brushing specimen ($P < .001$).

factors including extensive procedure time to perform the recommended biopsy protocol, inadequate sampling by the biopsy technique [3,20], and lack of pathologic reproducibility [7,21,22]. Cytology specimens collected via endoscopic brushing are less invasive, are less time intensive, and result in a more comprehensive sampling of the esophagus than is possible with the biopsy method. Ancillary cytologic techniques such as DIA and FISH could potentially be used on brushing specimens to improve the detection of dysplasia and adenocarcinoma in patients with BE.

Fahmy et al [23] used cytologic brushing specimens from BE to hybridize 2 FISH probe sets directed to (1) centromeric regions of chromosomes 6, 7, 11, and 12, and (2) 9p21 (*P16*), centromere 9, 17p13.1 (*P53*), and centromere 17. These probes had high sensitivity and specificity for the detection of HGD/EA; however, their ability to detect LGD was not tested. In comparison, the current study reveals that FISH using probes directed to 8q24 (*C-MYC*), 9p21 (*P16*), 17q12 (*HER2*), and 20q13 on endoscopic brushing specimens is more sensitive than both cytology and DIA for the detection of dysplasia (including LGD) and EA in patients with BE (Fig. 4). FISH analysis recognizes cells with minimal DNA alterations such as deletion of the 9p21 locus, which in our experience does not tend to cause noticeable morphological abnormalities. In addition, the FISH threshold for a positive result is low (eg, 4 polysomic cells) in comparison with cytology or DIA, which often require the detection of many abnormal cells to consider a case positive for abnormality.

Not only is FISH more sensitive than cytology or DIA; it can also identify different types of genetic abnormalities such as hemizygous and homozygous 9p21 loss, gain of 8q24, tetrasomy, and polysomy in the neoplastic cells. These genetic aberrations are likely to correspond with the severity of a lesion. A previous study with this probe set reported that the finding of 9p21 loss by FISH in brushing specimens suggests the patient has LGD [13]. Other investigators have shown that biopsies with LGD tend to be diploid or near

diploid lesions [24], and frequently show evidence of *P16* inactivation through allelic loss of the 9p21 region [12,24,25]. It should be noted that mechanisms such as point mutations or hypermethylation commonly lead to *P16* inactivation in BE, and that neither of these alterations are detectable by FISH [9,10,26,27]. In this study, most of the specimens (6/10, 60%) found to be positive by FISH with a corresponding biopsy showing LGD exhibited 9p21 loss. Much of the increased sensitivity of FISH compared with that of cytology and DIA for the LGD group of patients was due to the detection of 9p21 loss.

Fig. 5 illustrates that the fraction of specimens that show genetic abnormalities typical of HGD or EA (ie, single-locus gain, tetrasomy, polysomy) increases from 20% to 64% to 80% among the patients that were histologically diagnosed with LGD, HGD, and EA, respectively. A previous study with this probe set suggests that patients with a brushing specimen diagnosed as polysomy or single-locus gain are more likely to have HGD or EA, and that these lesions are not easily distinguishable by FISH alone [13]. This is likely due to the genetic similarity between HGD and EA [9,24].

Various investigators have used DNA ploidy analysis techniques such as flow cytometry and DIA to assess esophageal biopsy specimens for aneuploidy [24,28,29]. These studies have shown that most HGD and EA specimens are characterized by aneuploidy, whereas IM and LGD are typically diploid or near diploid. In fact, the finding of aneuploidy in a patient with a corresponding HGD diagnosis has been used as a criterion for aggressive intervention [28] because aneuploidy has been shown to be a better predictor than histology for the development of EA [29]. Unfortunately, these methods have not been widely adopted in a clinical setting because of technical demands, which has limited their availability to few medical institutions.

Although the type of chromosomal alteration detected by FISH in the endoscopic brushing specimen generally correlated well with the biopsy, there were some cases in which the chromosomal abnormalities detected by FISH were seemingly inconsistent with the biopsy findings. For example, 2 patients with IM and 3 patients with LGD had brushings that showed a polysomic signal pattern by FISH. It is possible that the brush sampled a higher-grade lesion than was sampled by biopsy. Mapping studies have shown that areas of HGD or EA may represent only a small fraction of the total area affected by BE [6,30], which could easily be missed by the biopsy sampling method.

Use of the tissue diagnosis as the gold standard for the evaluation of advanced cytologic techniques on brushing specimens has limitations, especially because of incomplete sampling. It is likely that small HGD/EA lesions not sampled by an initial biopsy would be detected on a follow-up visit. The patients in this study are from a high-risk population (ie, 91% have a history of HGD or EA) and return regularly for surveillance. Therefore, histologic results from endoscopic visits subsequent to the initial brushing specimen were used to create a Kaplan-Meier disease progression curve (Fig. 6).

Eighty-five percent of patients with a polysomic FISH result had HGD or EA at the time of the initial biopsy, and the remaining 15% progressed to HGD or EA within 6 months. For patients with 9p21 loss, 43% had HGD or EA on the initial biopsy and another 24% progressed within 4 months. In comparison, only 13% of patients with a negative FISH result had HGD/EA on the original biopsy, with an additional 18% progressing to HGD/EA after 22 months. This analysis indicates that regardless of the original biopsy diagnosis, patients with a polysomic FISH result are at highest risk for progression to HGD/EA, followed by patients with 9p21 loss and single-locus gain.

A limitation of this study is that there were few patients with a histologic diagnosis of benign squamous epithelium ($n = 14$) at the time of the brushing because of the high prevalence of disease in this population. This limitation may explain the fact that the specificity of "Cytology P + S + A" was unexpectedly the same as the specificity of "Cytology P + S." In our experience, the positive predictive value of an atypical cytology result for diagnosing cancer is poor (unpublished data). Therefore, the cytologic category that includes only suspicious and positive results ("Cytology P + S") as a positive test was used for statistical comparisons in the present study. This reasoning is supported by previous cytologic studies on BE, which did not subclassify specimens with reactive atypia but rather classified them as benign/negative [18,23,31].

The findings of this study suggest that a FISH assay using probes to 8q24, 9p21, 17q12, and 20q13 has higher sensitivity than DIA and cytology for the detection of dysplasia and EA in patients with BE while maintaining the high specificity of cytology. Analysis of follow-up data shows that patients with polysomy detected in a brushing specimen progress to HGD/EA significantly earlier than patients with other FISH abnormalities. The finding of 9p21 loss or gain of a single locus appears to be associated with a risk for progression that is less than patients with a polysomic result but significantly greater than those with a negative FISH result. Because of the less invasive nature of cell collection for this assay compared with the traditional biopsy protocol and the fact that test results stratify patients for risk of progression to severe lesions, FISH is a promising modality to augment current surveillance methods. Although this study has validated the FISH assay in a high-risk population, further studies in a general population are needed to determine the appropriate use of FISH results in clinical practice.

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