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Fluorescence in situ hybridization identifies high risk Barrett's patients likely to develop esophageal adenocarcinoma

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SUMMARY

Barrett's esophagus (BE) with high-grade dysplasia (HGD) defines a group of individuals at high risk of progression to esophageal adenocarcinoma (EA). Fluorescence in situ hybridization (FISH) has been shown to be useful for the detection of dysplasia and EA in endoscopic brushing specimens from BE patients. The aim of this study was to determine whether FISH in combination with histological findings would further identify more rapid progressors to EA. This is a retrospective cohort study of high-risk patients, having a history of biopsy-confirmed HGD without EA, with an endoscopic brushing specimen analyzed by FISH while undergoing endoscopic surveillance and treatment between April 2003 and October 2010. Brushing specimens were assessed by FISH probes targeting 8q24 (*MYC*), 9p21 (*CDKN2A*), 17q12 (*ERBB2*), and 20q13 (*ZNF217*) and evaluated for the presence of polysomy, defined as multiple chromosomal gains (displaying 3 signals for 2 probes). Specimens containing 4 cells exhibiting polysomy were considered polysomic. HGD was confirmed by at least two experienced gastrointestinal pathologists. Of 245 patients in this study, 93 (38.0%) had a polysomic FISH result and 152 (62.0%) had a non-polysomic FISH result. Median follow-up was 3.6 years (interquartile range [IQR] 2–5 years). Patients with a polysomic FISH result had a significantly higher risk of developing EA within 2 years (14.2%) compared with patients with a non-polysomic FISH result (1.4%, $P < 0.001$). These findings suggest that a polysomic FISH result in BE patients with simultaneous HGD identifies patients at a higher risk for developing EA compared with those with non-polysomy.

Keywords

Barrett's esophagus; cytology; esophageal adenocarcinoma; FISH; polysomy

INTRODUCTION

Barrett's esophagus (BE) is a change in the distal esophageal epithelium from stratified squamous epithelium to a specialized intestinal columnar type mucosa and is the best established risk factor for the development of esophageal adenocarcinoma (EA). Most patients with BE die from causes other than EA,¹ but the small fraction of BE patients that progress to EA are faced with a greater than 85% mortality.² Current guidelines concerning management of BE are primarily based on the presence and grade of dysplasia.³ Therapy, usually in the form of radiofrequency ablation, is recommended for Barrett's patients with high-grade dysplasia (HGD). However, this requires multiple treatments and some HGD patients can progress to EA during therapy. Identification of a rapidly progressing group is needed to direct therapy to more aggressive treatments such as widespread mucosal resection. Although these patients with HGD are less common, they represent the most important group of patients that can benefit from intervention as they have such a high rate of progression to invasive disease. The questionable prognostic value of histology is highlighted by a recent European controlled randomized trial in which progression rates to HGD/EA varied substantially from similar American trials.⁴ Use of an additional biomarker would allow for a more standardized and reproducible way to better define risk and allow for global comparisons.

Given the need to define risk, the prospect of utilizing biomarkers in addition to histology to aid clinicians in the management of patients with BE is attractive. EA progresses through a metaplasia-dysplasia-adenocarcinoma sequence. It is known that accumulating genetic alterations along with genetic instability are associated with this morphologic continuum, culminating in the development of EA.⁵ Biomarkers such as DNA ploidy, loss of heterozygosity for TP53 and CDKN2A, immunohistochemical analysis for TP53, CDKN2A, and Ki-67 expression and fluorescence in situ hybridization (FISH) have all been suggested as ways to stratify risk for developing EA in the setting of BE,⁶⁻⁸ but none of these biomarkers have been widely implemented in clinical practice.

FISH utilizes fluorescently labeled DNA probes to detect chromosomal alterations such as copy number alterations and chromosomal rearrangements in cells. FISH can be used to identify cells that have chromosomal abnormalities that are consistent with a diagnosis of neoplasia, both pre-malignant and malignant. A FISH probe set, consisting of probes targeting 8q24 (*MYC*), 9p21 (*CDKN2A*), 17q12 (*ERBB2*), and 20q13.2 (*ZNF217*), has been designed to identify genetic abnormalities in BE and BE-associated neoplasia.^{9,10} Studies utilizing this probe set on endoscopic brushing specimens from BE patients have demonstrated that the majority of patients with a histologic diagnosis of HGD and EA were found to have polysomic cells in their specimen.⁵ The current study was performed to determine whether FISH analysis of cytologic brushing specimens is able to identify which BE patients with HGD are at highest risk of developing EA. As more clinical therapies have been developed for ablation of HGD, it is more important than ever to be able to determine which of these patients need more aggressive treatment and careful endoscopic follow-up.

MATERIAL AND METHODS

Patients

This is a retrospective cohort study of BE patients who underwent endoscopic surveillance at the Mayo Clinic between April 2003 and October 2010. Inclusion criteria included FISH analysis on a brushing specimen collected during routine surveillance endoscopy, biopsy-confirmed HGD without EA prior to the endoscopy in which the brushing was collected, and a minimum of one follow-up visit. All patients had provided written informed consent under Institutional Review Board approved protocols (IRB 718-01, 2138-00, 2138-00, 1399-05, and 335-06). Clinical data were collected from the electronic medical record for the patients included in this study.

Specimen collection

Endoscopic brushing specimens were collected in conjunction with surveillance biopsy as previously described, except that a single gastrointestinal brush rather than multiple brushes were used for specimen collection.¹⁰ The mucosa surface was cleansed of mucus initially using 1% N-acetyl cysteine sprayed on the mucosa. This was then suctioned from the lumen of the esophagus and stomach. All visible areas of endoscopically recognizable Barrett's mucosa were sampled with the cytology brush. Nodular areas were brushed carefully. The mucosal surface was then biopsied in four quadrants every centimeter of affected mucosa using maximum capacity biopsy forceps which were submitted for histologic evaluation by experienced gastrointestinal pathologists. Subsequent surveillance intervals were based on current practice guidelines.³

Fluorescence in situ hybridization

FISH was performed using a four probe set of directly labeled DNA probes to 8q24 (*MYC*), 9p21 (*CDKN2A*), 17q12 (*ERBB2*), and 20q13.2 (*ZNF217*) (Abbott Molecular Inc., Des Plaines, IL, USA) on the brushing specimen as previously described and blinded to the histologic diagnosis of the corresponding biopsy.¹⁰ Specimens were analyzed by an experienced technologist who was blinded to all clinical data, including histologic diagnoses. FISH analysis included a 100 consecutive cell count followed by a scan for polysomic cells on specimens not meeting the threshold for positivity for polysomy within the 100 cell count.¹⁰ A specimen was considered positive for 9p21 loss if 6% of cells displayed homozygous loss (i.e. zero copies of the 9p21 probe) or 11% displayed either hemizygous loss (i.e. one copy of the 9p21 probe) or a combination of homozygous and hemizygous loss. The threshold of positivity for locus gain of any single locus or tetrasomy (i.e. four copies of all four probes) was 5%. The threshold of positivity for single locus loss of 8q24, 17q12, or 20q13.2 was 11%. A specimen showing multiple chromosomal gains (i.e. polysomy; displaying 3 signals for 2 probes) in four or more cells was considered polysomic (Fig. 1).

Statistical analysis

Patients were classified as 'polysomy' or 'non-polysomy' (e.g. 9p21 loss, single locus gain, disomy/normal), based on their FISH result. The most severe histologic diagnosis observed

in tissue collected during the same endoscopy as the brush was considered the baseline histologic diagnosis. Follow-up intervals and time-to-event analysis for progression to EA were calculated as the time between specimen collection and the first histologic diagnosis of EA or last endoscopy for patients who did not present with EA.

Associations between clinical and FISH findings and the risk of developing EA on follow-up were assessed with log-rank tests and univariate Cox proportional hazards regression models. The Kaplan–Meier method was used to estimate the risk of developing EA. To take into account the fact that therapy may not have occurred directly at baseline, as well as the fact that a single patient may have had multiple types of therapy throughout the course of their disease, the effect of therapy from baseline through follow-up was assessed as a time-dependent covariate considering endoscopic mucosal resection (EMR) and ablative therapies. Once a patient underwent EMR, they were entered into the EMR group, and once a patient underwent ablative therapy, they were entered in the ablation group. Hazard ratios for developing EA and 2-year risk estimates (along with 95% confidence intervals for each) were reported. Negative predictive value for non-polysomic FISH was calculated as 100% minus the 2-year risk of EA (EA-free survival) among those with a non-polysomic result. Positive predictive value for polysomic FISH was calculated as the 2-year risk of EA among those with a polysomic FISH result. *P*-values of <0.05 were considered statistically significant. Risk was summarized at 2 years because most patients in the data had at least 2 years of follow-up available. The total number of patients developing EA at any point in time is also reported. All analyses were performed using SAS version 9 (Cary, NC, USA).

RESULTS

This study included 245 BE patients with a history of biopsy-confirmed HGD without EA undergoing endoscopic surveillance at the Mayo Clinic (Table 1). The mean (standard deviation [SD]) age was 66 years (10), ranging from 35 to 90 years. The vast majority ($n = 209$, 85.3%) were male and 112 of the 245 (45.7%) patients had histological diagnosis of HGD from tissue collected during the same endoscopy as the brushing, 60 (24.5%) had low-grade dysplasia (LGD), and the remaining patients either had intestinal metaplasia (IM) or benign squamous epithelium (about 15% each) (Table 1). All patients had at least one follow-up endoscopy including histologic sampling after the procedure in which FISH was performed.

The median follow-up time was 3.6 years (interquartile range [IQR] 2–5 years), with 59.6% (146/245) of patients having 3 or more years of follow-up data. Twenty-four patients developed EA during follow-up. The estimated risk of developing EA within 2 years from baseline was 6.1% (95% CI: 3.0% to 9.2%). The majority of patients that developed EA presented with intramucosal cancer ($n = 18$, 75.0%) as opposed to submucosal cancer ($n = 6$, 25.0%).

Ninety-three of the 245 (38.0%) patients had a polysomic FISH result and 152/245 (62.0%) patients had a non-polysomic FISH result (Table 2). Among the 152 patients without polysomy, 107 (70.4%) had no abnormalities, 21 (13.8%) had 9p21 loss, 21 (13.8%) had single locus gain, 2 (1.3%) had tetrasomy, and 1 (0.7%) had single locus loss (Table 2). The

risk of EA was significantly higher among patients with a polysomic result compared with those without a polysomic result (Table 2). Patients with a non-polysomic result had an estimated risk of 1.4% (95% CI: 0% to 3.3%) of developing EA within 3 years (unchanged between years 1–3), as opposed to polysomic patients' risk of 10.2% (95% CI: 3.9% to 16.6%) within 1 year, 14.2% (95% CI: 6.7% to 21.7%) within 2 years, and 20.4% (95% CI: 11.3% to 29.5%) within 3 years (Fig. 2). In this high-risk patient cohort, in whom all patients had a prior history of HGD, the negative predictive value of a non-polysomic FISH result is estimated to be 98.6% at 2 years after brushing collection (100–1.4% 2-year risk noted above). Further, the positive predictive value of a polysomic FISH result is estimated to be 14.2% within 2 years.

The risk of developing EA was not significantly associated with gender, age, or Barrett's segment length (Table 2). There was no association between a polysomic FISH and a non-polysomic FISH result and the stage of cancer ($P = 0.57$). Of 19 patients with a polysomic FISH result, 15 (78.9%) presented with intramucosal cancer and 4 (21.1%) with submucosal cancer. Submucosal cancers are known to have an increased risk of metastatic disease. Of the five patients with a non-polysomic FISH result, three (60.0%) presented with intramucosal cancer and two (40.0%) with submucosal cancer. Although all patients had a history of HGD, simultaneous histology (collected at the time FISH was performed) did affect outcomes. There was an increased risk of progressing to EA as the severity of the simultaneous histologic diagnosis increased with 2-year risk estimates of 0%, 2.9%, 3.8%, and 10.4% for absence of benign squamous epithelium, IM, LGD, and HGD, respectively (Table 2). Patients with simultaneous HGD were more likely to develop EA than those that did not have HGD on biopsy (HR = 4.5, 95% CI: 1.8–11.5, 2-year risk estimates 10.4% vs. 2.6%, $P = 0.0005$). The majority of patients with HGD ($n = 112$) also had polysomy (63.4%), which was associated with the highest risk of developing EA (2-year risk estimate 15.2%). When considering all polysomy and baseline HGD combinations together, those with both had the highest risk of EA compared with those with neither (HR = 11.8, 95% CI: 3.4–40.9) (Table 2 and Fig. 3). However, in a model that included both polysomy and HGD, only polysomy remained significant ($P = 0.002$).

The majority of patients (84.8%, 207/244) underwent endoscopic therapy during the baseline procedure and/or at some point during the course of follow-up (note, one patient who underwent chemotherapy at baseline was excluded from the therapy analysis). Of the 207 patients having endoscopic therapy, 81 (39.1%) underwent EMR only (either at baseline or during follow-up) while 126 (60.9%) had ablative therapy at baseline or during follow-up (regardless of EMR treatments). Overall, therapy was associated with development of EA ($P = 0.0003$). However, in a separate cox proportional-hazards regression model that adjusted for therapy, polysomy remained statistically significant (HR = 6.2, 95% CI: 2.7–17.0, $P = 0.0004$) for the development of EA while therapy was no longer significant.

CONCLUSIONS

We have developed a FISH assay consisting of probes to 8q24 (*MYC*), 9p21 (*CDKN2A*), 17q12 (*ERBB2*), and 20q13.2 (*ZNF217*) that has been shown to be useful for the detection of dysplasia and EA in endoscopic brushing specimens from patients with BE.¹⁰ A variety

of molecular techniques and markers, including DNA ploidy, proliferation markers (TP53, MIB-1, and Ki-67), and FISH, have been evaluated for their ability to detect dysplasia and EA in patients with BE.^{10,11} However, very few have investigated the utility of these markers for predicting which patients with dysplasia progress to EA. Our current study illustrates how FISH can be utilized to assess the likelihood of developing EA in high-risk BE patients.

A clinical assay that could identify patients at higher risk of developing EA would be beneficial to physicians for selection of therapeutic options. Current recommendations include ablation therapy for HGD patients. Patients identified as having a higher risk of developing EA might benefit from more aggressive treatment options (e.g. widespread mucosal resection), more rapid or sequential ablations, or a change in therapies if these patients do not respond rapidly.

In our study, high-risk BE patients with a polysomic FISH brushing result were significantly more likely to develop EA compared with those with a non-polysomic result ($P < 0.0001$, Fig. 2) and had an estimated risk of 14.2% of developing EA within 2 years, as opposed to a risk of 1.4% for patients with a non-polysomic FISH result. Therefore, FISH may have clinical utility for identifying HGD patients who are less likely to progress to EA (i.e. non-polysomic FISH result). Risk stratification by FISH could identify HGD patients at lower risk of progression. Such patients could benefit from a less aggressive treatment approach, especially in the context of other co-morbidities.

While the absence of polysomy had a high negative predictive value for progression to EA (98.6% at 2 years), there were five non-polysomic patients that developed EA in this study, three showed no abnormalities by FISH, and the remaining two displayed single locus gain. Possible explanations for progression to EA in non-polysomic patients include inadequate sampling (i.e. the endoscopic brushing did not sample the entire lesion) and genetic alterations undetectable by this assay. Identification of genetic alterations was limited to chromosomal gain/loss of genes targeted by the FISH probes utilized in this study (*MYC*, *CDKN2*, *ERBB2* and *ZNF217*). Therefore, other genetic alterations that may be driving the tumor (e.g. point mutations, methylation, or chromosomal gain/loss other than those targeted in the probe set) would not be identified.

Our data indicate that patients with a baseline histologic diagnosis of HGD were 4.5 times more likely at a given point in time to develop EA than patients with less severe histologic results (HR = 4.5). Similarly, patients with polysomic FISH result were 7.9 times more likely to develop EA than patients with non-polysomy results (HR = 7.9). Furthermore, the subset of patients with both HGD at baseline and a polysomic FISH result had over twice the risk of EA compared with those with either HGD (HR = 5.7) or polysomy alone (HR = 2.1). This suggests that both are important predictors of determining who will develop EA; however, only the effect of polysomy was significant ($P = 0.002$) in a multivariable model that also included HGD at baseline. Of the two patients with a baseline histologic diagnosis of HGD and a non-polysomic FISH result who developed EA, one patient was found to have a polysomic FISH result and EA histology at the first follow-up visit (119 days) while the other patient had a polysomic FISH result at the time of EA diagnosis (1606 days).

We recognize that our Radiofrequency ablation (RFA) failure rate is somewhat higher than other reported rates. This could be attributed to the effect of referral bias in that our patients are more likely to have longer segments of Barrett's mucosa and/or prior RFA failure. While the results of this study show promise for aiding in risk assessment for the development of EA in patients with a history of HGD, any conclusion from these analyses, particularly those which included more than one predictor simultaneously (i.e. grade and polysomy, therapy and polysomy), should be made cautiously given the small number of events ($n = 24$). One could argue that patients progressing to EA within a year may have had EA at baseline that was not detected by biopsy and therefore should be excluded from analysis.¹² However, if patients who either developed EA within the first year or had less than 1 year of follow-up are excluded from the analysis, our findings are unchanged. The 2-year risk estimates for EA in this subset were 4.4% and 0% for polysomy and non-polysomy, respectively ($P = 0.0002$).

There is an ever-growing need for biomarkers to aid in the risk stratification of patients with BE, especially for patients who have been diagnosed with HGD. Our findings suggest that in a high-risk population of BE patients, those with polysomic FISH brushing results are at greater risk of developing EA compared with those without polysomy. Patients with a polysomic FISH result should be monitored closely. The high negative predictive value of a non-polysomic FISH result for the development of EA may justify less aggressive treatment or observation in this patient group. Additional multi-institutional prospective studies are needed to confirm the findings of this study and to address the role of FISH analysis in predicting risk of progression to EA in a lower-risk BE population, including patients with LGD and non-dysplastic BE.

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Fig. 1. Representative examples of fluorescence in situ hybridization signal patterns observed in cells from esophageal brushing specimens. (a) Non-polysomic; (b) polysomic (3 signals for 2 probes) signal patterns observed in cells obtained from esophageal brushing specimens. FISH probes: 8q24 (aqua), 9p21 (red), 17q12 (green), and 20q13.2 (gold).

Fig. 2. Kaplan–Meier survival estimates according to the presence or absence of polysomy detected by fluorescence in situ hybridization on endoscopic brushing specimens collected at baseline ($P < 0.0001$).

Fig. 3. Kaplan–Meier failure plot demonstrating risk of esophageal adenocarcinoma (EA) among patients with a baseline histologic diagnosis of high-grade dysplasia (HGD) based on the corresponding fluorescence in situ hybridization (FISH) brushing results. The groups shown are not mutually exclusive. $P = 0.01$ for polysomic versus non-polysomic FISH.

Table 1

Patient characteristics

Variable	Total (n = 245)
Age, years	
Mean (standard deviation)	66.6 (10.0)
Range	34.6–90.3
Sex	
Female	36 (14.7%)
Male	209 (85.3%)
Barrett's segment length (baseline)	
Mean (standard deviation)	4.0 (3.7)
<3 cm	108(44.1%)
3 cm	137 (55.9%)
Histology diagnosis * (baseline)	
Benign squamous epithelium	36 (14.7%)
Intestinal metaplasia without dysplasia	37(15.1%)
Low grade dysplasia	60 (24.5%)
High grade dysplasia	112(45.7%)
Progression to esophageal adenocarcinoma	
Yes, n (2-year Kaplan–Meier estimate)	24(6.1%)
No	221 (93.9%)
Total time of follow-up (year)	
Median	3.6
Q1, Q3	2.0, 5.0
Range	0.2–7.8

* Histologic diagnosis was classified as the most severe diagnosis observed per biopsy/endoscopic mucosal resection (EMR) specimens taken during baseline endoscopy.

Table 2

Results of time to esophageal adenocarcinoma analysis

Variable	No. of patients	Patients developing EA, <i>n</i> (2-year Kaplan–Meier estimate)	Univariate HR (95% CI)	Log-rank <i>P</i> -value
Sex				
Female	36	2 (2.9%)	Reference	0.40
Male	209	22 (6.6%)	1.84 (0.43, 7.84)	
Age, years				
Per-year increase	245		1.00 (0.96, 1.04)	0.98
Barrett's segment length (baseline)				
<3 cm	108	8 (5.2%)	Reference	0.34
≥3 cm	137	16 (6.8%)	1.51 (0.64, 3.52)	
Histology diagnosis [†] (baseline)				
Benign squamous epithelium	36	0 (0%)	NA	0.003
Intestinal metaplasia without dysplasia	37	1 (2.9%)	0.30 (0.04, 2.59)	
Low grade dysplasia	60	5 (3.8%)	Reference	
High grade dysplasia	112	18 (10.4%)	2.33 (0.87, 6.30)	
FISH abnormality				
None	107	3 (2.0%)	Reference	<0.0001
9p21 Loss	21	1 (0%)	1.54 (0.16, 14.78)	
Single locus gain	21	1 (0%)	1.67 (0.17, 16.08)	
Polysomy	93	19 (14.2%)	9.06 (2.67, 30.72)	
Polysomy FISH				
No	152	5 (1.4%)	Reference	<0.0001
Yes	93	19 (14.2%)	7.93 (2.95, 21.31)	
High grade dysplasia (baseline)				
No [‡]	133	6 (2.6%)	Reference	0.0005
Yes	112	18 (10.4%)	4.54 (1.79, 11.48)	
Polysomy FISH and high grade dysplasia (baseline)				
No	174	8 (2.5%)	Reference	<0.0001
Yes	71	16 (15.2%)	6.63 (2.82, 15.61)	
FISH and histology diagnosis (baseline)				
Non-polysomy and non-HGD	111	3 (1.0%)	Reference	<0.0001
Non-polysomy and HGD	41	2 (2.4%)	2.08 (0.35, 12.46)	
Polysomy and non-HGD	22	3 (10.8%)	5.61 (1.13, 27.81)	
Polysomy and HGD	71	16 (15.2%)	11.83 (3.42, 40.87)	

[†] Histologic diagnosis was classified as the most severe diagnosis observed per biopsy/EMR specimens taken during baseline endoscopy.

[‡] Non-HGD includes (benign squamous epithelium, intestinal metaplasia without dysplasia, and low-grade dysplasia). HGD, high-grade dysplasia.