Original Article

A biomarker panel predicts progression of Barrett's esophagus to esophageal adenocarcinoma

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SUMMARY. Progression from Barrett's esophagus (BE) to esophageal adenocarcinoma (EAC) is uncommon but the consequences are serious. Predictors of progression are essential to optimize resource utilization. This study assessed the utility of a promising panel of biomarkers applicable to routine paraffin embedded biopsies (FFPE) to predict progression of BE to EAC in a large population-based, nested case-control study.

We utilized the Amsterdam-based ReBus nested case-control cohort. BE patients who progressed to high-grade dysplasia (HGD)/EAC ($n = 130$) and BE patients who never progressed ($n = 130$) were matched on age, sex, length of the BE segment, and duration of endoscopic surveillance. All progressors had minimum 2 years of endoscopic surveillance without HGD/EAC to exclude prevalent neoplasia. We assessed abnormal DNA content, p53, Cyclin A, and Aspergillus oryzae lectin (AOL) in FFPE sections. We performed conditional logistic regression analysis to estimate odds ratio (OR) of progression based on biomarker status.

Expert LGD (OR, 8.3; 95% CI, 1.7–41.0), AOL (3 vs. 0 epithelial compartments abnormal; OR, 3.6; 95% CI, 1.2–10.6) and p53 (OR, 2.3; 95% CI, 1.2–4.6) were independently associated with neoplastic progression. Cyclin A did not predict progression and DNA ploidy analysis by image cytometry was unsuccessful in the majority of cases, both were excluded from the multivariate analysis. The multivariable biomarker model had an area under the receiver operating characteristic curve of 0.73.

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Conflicts of interest: Jacques J.G.H.M. Bergman receives research support from Olympus Endoscopy, Cook Medical, Boston Scientific, GI Solutions Covidien, Erbe and Ninepoint Medical; receives financial support for training programs from GI Solutions Covidien; and receives honorarium-consultancy-speakers fee from Cook Medical, Boston Scientific and Covidien GI Solutions (Medtronic). Rebecca C. Fitzgerald has named patents related to biomarkers for risk stratification and has patented Cytosponge-biomarker technology which has been licensed to Covidien GI Solutions (Medtronic). Nicholas J. Shaheen receives research support from Interpace Diagnostics, CDx Medical, CSA Medical, GI Solutions Covidien, and Takeda Pharmaceuticals. W. Asher Wolf has received support to attend educational events from Nestlé. All other authors have no conflicts of interest to declare.

Funding: This work was funded in part by grants from the American Society of Gastrointestinal Endoscopy, as well as the National Institutes of Health (U54CA163060, T32 DK07634, and P30 DK 034987).

Expert LGD, AOL, and p53 independently predict neoplastic progression in BE patients and are applicable to routine practice. These biomarkers can aid in selecting patients for endoscopic ablation or more intensive surveillance.

KEY WORDS: Barrett's esophagus, biomarkers, esophageal adenocarcinoma, neoplastic progression.

INTRODUCTION

In Barrett's esophagus (BE), columnar epithelium containing goblet cells has replaced the normal squamous epithelium lining the distal esophagus.¹ BE patients are at increased risk of developing esophageal adenocarcinoma (EAC), often preceded by dysplasia.^{1–3} The annual rate of progression from non-dysplastic BE (NDBE) to high-grade dysplasia (HGD) or EAC is relatively low, ranging from 0.2 to 0.5% per year.^{4–6} Since a diagnosis of invasive EAC has a dismal prognosis, with an overall 5-year survival of 20%, BE patients are offered regular endoscopic surveillance to detect neoplasia at a curable stage.^{3,7–9} Patient management is based on the endoscopic assessment and the histopathological evaluation of surveillance biopsies. This approach has several shortcomings, such as questionable cost-effectiveness, difficulties with identifying dysplasia endoscopically, biopsy sampling error, and interobserver variation between pathologists for diagnosing dysplasia.^{10–13} A biomarker panel that could objectively stratify BE patients according to their risk of progression to HGD/EAC would, therefore, considerably improve BE surveillance. Such a panel could help target expensive therapeutic interventions or surveillance endoscopies at high-risk individuals, whereas patients at low risk of neoplastic progression could be surveyed at lower frequency or be discharged from surveillance entirely.

Several previous studies have identified potential biomarkers for progression in BE. Alterations in DNA copy number as well as specific chromosomal abnormalities have been demonstrated to be associated with neoplastic progression.^{14–16} Epigenetic markers such as CpG island promotor hypermethylation of *p16*, *RUNX3* and *HPPI* have also been indicated to predict progression risk.^{17,18} Mutation of the *p53* gene, detectable as complete loss or overexpression of the p53 protein on immunohistochemistry, has been extensively investigated and identified as a marker of neoplastic progression in several studies.^{19–21}

However, most of these markers have limited clinical applicability due to issues with biomarker assessment or lack of sufficient validation. In order for a biomarker panel to be clinically applicable, the assay should ideally be performed on tissue material that is readily available in the clinical setting such as formalin

fixed paraffin embedded biopsies. The need for fresh frozen biopsy material as well as special equipment and interpretation complicates implementation in the setting of community BE surveillance.

Additionally, many phase 3 studies (retrospective longitudinal studies) on biomarkers for BE progression suffer methodological shortcomings. Patients are often identified in tertiary care referral centers, which can limit generalizability of the findings.^{14,16,17,19} Furthermore, the selection criteria for progressor patients in most of the previous studies have been variable. To adequately assess a biomarker's ability to predict future risk of neoplastic progression, progressor patients with prevalent disease at baseline must be excluded. Studies have included progressor patients who developed HGD/EAC within a certain interval after the baseline NDBE diagnosis, yet in most studies this interval has been relatively short (generally set at 6 months).^{14,15,17–19,21–23} The possibility of selecting patients with prevalent disease at baseline cannot be excluded with such a relatively short surveillance interval. Additionally, the stage of disease at time of progression is also relevant to exclude potential prevalent neoplasia at the baseline endoscopy. In progressor patients with advanced cancer ($\geq T2$), even a long interval (>2 years) between baseline and cancer diagnosis may not suffice and therefore these patients should preferably be excluded, which has often not been done.^{14,15,18,19,21–23}

Recent work has identified two panels of biomarkers in BE to predict future risk of progression and prevalent dysplasia, respectively.^{15,24} The first panel includes a consensus diagnosis of LGD, aneuploidy, and aspergillus oryzae lectin (AOL) immunohistochemistry (IHC), which was applied to a retrospective nested case-control study (North Ireland Barrett's Registry).¹⁵ The adjusted OR for progression for each additional positive biomarker in the reduced model was 3.91 (95%CI 2.39–6.37) and 3.31 (95%CI 1.81–6.05), depending on whether a consensus diagnosis of LGD was or was not present respectively. The second panel, which comprises aneuploidy, p53 (IHC), and cyclin A (IHC), was tested in a multicenter prospective study.²⁴ This panel predicts inconspicuous prevalent HGD/EAC with a sensitivity of 100% and a specificity of 85%. A combination of these two panels (total of five biomarkers) might therefore be able to identify high-risk patients who harbor prevalent dysplasia and who are at high risk

for future progression to cancer. This panel requires further independent validation and reducing to the smallest number of variables possible to maximize future clinical utility.

The aim of this study is to assess the performance of this 5-biomarker panel in an independent nested case-control cohort of patients with BE with or without low-grade dysplasia who progressed to cancer (progressors) compared to those who did not (nonprogressors). This study was performed in the community-based Amsterdam ReBus cohort, which includes a stringently selected population of BE progressors and nonprogressors as outlined in the methods section.

METHODS

Population and setting

The Amsterdam ReBus cohort consists of BE patients who progressed to HGD/EAC during endoscopic surveillance (progressors) and those who never demonstrated neoplastic progression (nonprogressors). Progressors were identified in three tertiary care referral centers in the Netherlands. For all patients who were referred from community centers for expert work-up of neoplasia between 2000 and 2012, a detailed endoscopic surveillance history was performed. The nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA database) was used to identify all surveillance endoscopies with biopsies that were performed. The PALGA database has nationwide coverage since 1991, archiving the reports of all pathology laboratories in the Netherlands.²⁵ Subsequently, the original endoscopy and pathology reports were retrieved from the referring hospitals and detailed information on the surveillance history was recorded. Nonprogressors were identified from a retrospective BE surveillance registry in 10 community hospitals in the Amsterdam region. The retrospective registration was initiated in 2003 and was updated from 2011 onwards.²⁶ This second analysis enabled us to identify newly diagnosed BE patients and yielded valuable follow-up information on patients who were identified in the previous analysis. Inclusion criteria were: baseline endoscopy demonstrating columnar lined esophagus with specialized intestinal metaplasia on subsequent histological examination; minimum two years endoscopic surveillance before the endpoint of the study; maximum stage T1 disease at time of progression (progressors); progression diagnosis based on an endoscopic resection (ER) or esophagectomy specimen or biopsies obtained at two separate endoscopies (progressors).

The endpoint of this study was development of HGD or EAC (progressors) or the latest known

endoscopy with histological assessment of biopsies demonstrating at most LGD (nonprogressors).

Progressors and nonprogressors were matched based on sex, age (± 5 years), length of the BE segment (± 2 cm), and duration of endoscopic surveillance (± 2 years).

The study was reviewed by the institutional ethics committee of the Academic Medical Center (AMC), Amsterdam. The institutional biobank review committee of the AMC officially approved the ReBus biorepository.

Histopathology and selection of tissue material

Biopsies were formalin fixed and paraffin embedded at each local hospital according to different local fixation and embedding protocols. All outcome biopsies or ER specimens in progressor patients were reviewed by at least two expert pathologists to confirm the HGD or EAC diagnosis. Additionally, expert histological review was performed for all index biopsies demonstrating indefinite for dysplasia (IND) or LGD, in order to assess for LGD. Histological scoring was performed according to the Vienna classification.²⁷

For each patient, one cassette of formalin fixed paraffin embedded biopsies was selected with a histological diagnosis of NDBE or LGD at most. We selected biopsies that were obtained at least 2 years before the endpoint of the study as described above and when available, a three to five year interval was chosen.

Image cytometry DNA analysis

Samples ($4 \times 10 \mu\text{m}$ sections) were deparaffinized in xylene followed by rehydration in decreasing concentrations of ethanol. The samples were then incubated in 2.5 mg/mL proteinase XXIV in PBS overnight. The extracted nuclei were strained using a $30 \mu\text{m}$ mesh strainer and stained with DAPI. The samples were plated in a 96-well plate then spun down before being scanned at $\times 200$ magnification using the iCys (CompuCyte) laser scanning cytometer version 7.0. A histogram representing the DNA content was produced and analyzed using the Flowjo 7.6 software according to European Society for Analytical Cellular Pathology guidelines.²⁸ All histograms were analyzed blindly by two observers (NG-D and SZ).

Histo- and immunohistochemistry and scoring

Histochemistry was performed for p53 (1/50 dilution, retrieval H1 30 min, DO7, Leica, Milton Keynes), Cyclin A (1/50 dilution, retrieval H1 10 min, Leica) and AOL (5 $\mu\text{g/mL}$; Tokyo Chemical Industry, UK labeled with Proton Biotin labeling kit from Vector) on $4 \mu\text{m}$ section using the BOND autostainer (Leica,

Milton Keynes, UK) following manufacturer instructions. Stained sections were then counterstained with hematoxylin.

p53 intensity was scored on a 0–3 scale with scores of 0 and 3 abnormal. A p53 score of 0 refers to the so-called ‘absent’ pattern where there is a complete lack of p53 staining in comparison to the background ‘normal’ wild-type staining in the glandular epithelium elsewhere in the biopsy.²⁹ The percentage of cyclin A positive compared to negative epithelial surface cells was calculated. A cutoff of 1% was used for significance.³⁰

AOL was graded for intensity (0–3) and for the % of the area stained at this intensity (0–4: 1 is 1–25%, 2 is 26–50%, 3 is 51–75%, 4 is > 75%). The H score (0–12) derived by multiplying intensity and area scores was calculated (0–12) and provides more accurate scoring compared to either the intensity or area score alone.³¹ Three epithelial compartments were assessed: the apical epithelial membrane, pan membranous, and epithelial mucous globule (globular collections of staining within the cytoplasm). The sample was abnormal if the product of the surface area and intensity was <4. IHC scoring was blinded to progressor status. Representative histo- and immunohistochemistry stains are demonstrated in supplementary Figure S1.

Statistical analysis

Continuous variables with a normal distribution were described with mean and standard deviation. Median and interquartile range were used to describe continuous variables with a skewed distribution. Bivariate testing compared progressors and nonprogressors using paired t-test for normally distributed continuous variables, Wilcoxon signed rank for non-normally distributed continuous variables, and McNemar’s test for categorical variables. Conditional logistic regression was used to estimate odds ratios (OR) of progression based on each biomarker independently (bivariate regression) and for multiple biomarkers together (multivariate regression). These models were further adjusted for potential confounders. We calculated the C statistic (area under the receiver operating characteristics curve) to evaluate the discriminatory performance of the multivariable predictive model. All reported *P*-values were two-tailed and *P*-values of <0.05 were considered to indicate statistical significance.

Statistical calculations were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Patients

Two hundred sixty patients (130 progressors and 130 nonprogressors) met inclusion criteria (see Fig. 1 for exclusions). Despite matching for length of the BE

segment (margin of 2 cm), progressor patients had a slightly, but significantly longer BE segment (5 cm, IQR 4 to 7) compared with nonprogressors (4 cm, IQR 3 to 6; *P* < 0.001). Mean duration of surveillance in this study (3.7 vs. 4.8 years; *P* < 0.001) was shorter in progressors than in nonprogressors. Progressors and nonprogressors did not differ by the remaining matching criteria (age and sex). Following expert consensus histology review of all LGD cases, baseline histology was predominately NDBE (*n* = 257, 91%), while a small number of patients had confirmed LGD (*n* = 18, 6%) or IND (*n* = 8, 3%). Progressors were more often diagnosed with confirmed LGD at baseline (12% vs. 2%; *P* < 0.001). Baseline characteristics of the study population are summarized in Table 1.

Neoplastic progression

Individual biomarkers

A diagnosis of LGD, independently confirmed by two expert pathologists, was associated with a 34-fold increased odds of progression to HGD/EAC (Table 2). Among 74 patients with abnormal expression of p53, 63 patients (85%) had overexpression of p53 and 11 patients (15%) had absent staining. Abnormal expression of p53 significantly predicted neoplastic progression on bivariate analysis (OR, 3.0; 95% CI, 1.1 to 7.9). While abnormal AOL expression did not significantly predict progression, an abnormal AOL score for three cell compartments did show a trend toward increased odds of progression (OR, 4.1; 95% CI, 0.8 to 20.6). Cyclin A abnormalities were not associated with future development of HGD/EAC. The bivariate odds ratios for the individual biomarkers are depicted in Table 2.

Despite multiple attempts to optimize the protocol the analysis of DNA content abnormalities by image cytometry proved to be unsuccessful in the vast majority of patients, yielding insufficient cell counts (supplementary Fig. S2). Therefore, this biomarker was excluded from further analyses.

Multivariable biomarker panel

We combined expert LGD, AOL, and p53 (cyclin A was excluded due to lack of effect on bivariate analysis) into a multivariable predictive model. We then further adjusted this model for potential clinical confounding variables (current tobacco and alcohol use, BE segment length, and time in study). In the multivariate analysis, expert LGD had a 36-fold increased odds of progression (OR, 35.7; 95% CI, 1.4–920.8). Abnormal p53 expression (OR, 4.1; 95% CI, 1.4–12.4) and abnormal AOL expression in three epithelial compartments (OR, 4.3; 95% CI, 0.7–26.3) had a 4-fold increased odds of progression (Table 3). This biomarker panel was able to discriminate well between progressors and nonprogressors with a C statistic of

Fig. 1 Flowchart of exclusions and matching of progressors ($n = 130$) and nonprogressors ($n = 130$) in this study. *11 patients were unpaired prior to the exclusions outlined above ($n = 5$ progressors and 6 nonprogressors). An additional 12 patients became unmatched because their partners were excluded for the reasons detailed above ($n = 2$ progressors and 10 nonprogressors).

0.73 (Fig. 2). Table 4 depicts sensitivity and specificity estimates for three evenly spaced cutpoints.

DISCUSSION

This community-based nested case-control study demonstrates that previously identified biomarkers can help predict neoplastic progression in BE patients. An expert LGD diagnosis (OR, 35.7), abnormal p53 expression (OR, 4.1), and abnormal expression of AOL (OR, 4.3) were all independently associated with progression to HGD/EAC. These biomarkers could help stratify BE patients according to their risk of progression and tailor decisions on prophylactic ablation therapy or endoscopic surveillance.

This study investigated a panel of biomarkers that was identified previously in a patient cohort from the Northern Ireland Barrett's Registry.¹⁵ The individual

biomarkers as well as the multibiomarker model had similar predictive ability in this study (Table 5). Given the modest AUC for the multibiomarker model in both studies, caution should be exercised in the interpretation of the biomarker panel outcome. Decisions on endoscopic surveillance intervals or prophylactic treatment cannot be recommended based on our biomarker panel, prior to validation of these markers in future phase 4 and 5 studies.

DNA content abnormalities have previously been shown to be highly associated with neoplastic progression in BE.^{15,32,33} Two previous studies have also demonstrated that image cytometry can accurately detect abnormalities in DNA content (aneuploidy/tetraploidy) using 40 μ m sections from paraffin embedded BE biopsies.^{15,34} Despite these previous results, image cytometry analysis did not yield sufficient cell counts in the majority of patients in this study, precluding a reliable assessment of DNA

Table 1 Baseline characteristics of the study population (*n* = 260)

Characteristic	Progressors <i>n</i> = 130	Nonprogressors <i>n</i> = 130	<i>P</i> -value
Age, years $\pm$ SD [†]	59.9 $\pm$ 9.6	59.2 $\pm$ 9.7	0.11
White race, <i>n</i> (%) [‡]	85 (100)	113 (97)	NA [§]
Male, <i>n</i> (%) [†]	104 (80)	105 (81)	0.32
Body mass index (IQR)	27.2 (24.9, 30.1)	26.1 (24.4, 28.7)	0.01
Current smoker, <i>n</i> (%)	18 (17)	17 (13)	0.14
Current alcohol, <i>n</i> (%)	64 (59)	94 (73)	0.04
Barrett's segment length, cm (IQR) [†]	5 (4, 7)	4 (3, 6)	0.02
Time in study, years (IQR) [†]	3.7 (2.9, 4.5)	4.8 (3.6, 5.8)	<0.001
Baseline histology [¶]			0.002
Non-dysplastic Barrett's esophagus, <i>n</i> (%)	108 (83)	127 (97)	
Indefinite for dysplasia, <i>n</i> (%)	7 (5)	1 (1)	
Low-grade dysplasia, <i>n</i> (%)	16 (12)	2 (2)	

[†]Progressors and nonprogressors were matched for these variables;[‡]Ethnicity was self-reported and 58 patients did not have a recorded value;[§]Paired testing produces no value because all progressors were from a single category (white);[¶]Expert histological review was performed in all cases with baseline low-grade dysplasia.**Table 2** Crude and adjusted bivariate odds of neoplastic progression for the individual biomarkers

Marker	Progressors <i>n</i> (%)	Nonprogressors <i>n</i> (%)	Unadjusted OR [95% CI]	Adjusted [†] OR [95% CI]
Expert dysplasia				
No LGD	115 (88)	128 (98)	1 (referent)	1 (referent)
LGD	15 (12)	2 (2)	7.5 [1.7, 32.8]	34.3 [3.4, 350.5]
p53 [‡]				
Negative	73 (60)	102 (80)	1 (referent)	1 (referent)
Abnormal	49 (40)	25 (20)	2.8 [1.5, 5.1]	3.0 [1.1, 7.9]
p53 three groups [‡]				
Wild type	73 (60)	102 (80)	1 (referent)	1 (referent)
No staining	8 (7)	3 (2)	3.0 [0.8, 11.4]	5.6 [0.7, 43.9]
Overexpression	41 (34)	22 (17)	2.7 [1.4, 5.4]	4.6 [1.8, 11.9]
Cyclin A [§]				
Negative	63 (76)	73 (74)	1 (referent)	1 (referent)
Positive	20 (24)	26 (26)	0.9 [0.4, 1.8]	0.9 [0.2, 3.3]
AOL [¶]				
0 compartments abnormal	24 (20)	36 (31)	1 (referent)	1 (referent)
1 compartments abnormal	37 (31)	48 (41)	1.0 [0.5, 2.1]	0.7 [0.3, 1.9]
2 compartments abnormal	35 (30)	27 (23)	1.4 [0.7, 2.9]	1.3 [0.5, 3.4]
3 compartments abnormal	22 (19)	7 (6)	5.3 [1.78, 17.3]	4.1 [0.8, 20.6]

[†]Adjustments were made for current tobacco and alcohol use, Barrett's segment length, and time in follow-up. All models except the model for LGD are further adjusted for baseline dysplasia;[‡]11 patients with low quality staining excluded;[§]78 patients with low quality staining excluded;[¶]24 patients with low quality staining excluded.

content abnormalities. It is likely that heterogeneous protocols for formalin fixation and paraffin embedding of biopsies affected the image cytometry processing since samples came from 32 different hospitals. Delay in paraffin embedding of the biopsies, resulting in increased duration of formalin fixation, was likely an important factor in divergent DNA quality of samples. In addition there may have been differences in adherence to the Seattle biopsy protocol and size of forceps used across participating centers, resulting in variable tissue quantities. The precise reason for the lack of a consistent and reproducible assessment of DNA content abnormalities using image cytometry could not be identified.

While the biomarker model in the previous study included ploidy analyses, the current biomarker model did not. Since ploidy measurements are complicated and time-consuming protocols, the current model would be clinically even more easily applicable without compromising predictive ability. New, promising methods for assessing abnormal DNA ploidy on formalin fixed material have been suggested, such as ultralow depth whole genome sequencing and single-nucleotide polymorphism arrays.^{35,36} These potentially simpler and clinically applicable methods for assessing DNA copy number abnormalities should be investigated as an addition to the current panel for assessment of risk of neoplasia in BE.

Table 3 Adjusted multivariate odds of neoplastic progression for the multibiomarker panel

Marker	Progressors <i>n</i> (%)	Nonprogressors <i>n</i> (%)	Adjusted [†] OR
Expert dysplasia [‡]			
No LGD	79 (87)	108 (99)	1 (referent)
LGD	12 (13)	1 (1)	35.7 [1.4, 920.8]
p53			
Negative	53 (58)	88 (81)	1 (referent)
Abnormal	38 (42)	21 (19)	4.1 [1.4, 12.4]
p53 three groups			
Wild type	53 (58)	88 (81)	1 (referent)
Absent staining	6 (7)	2 (2)	5.4 [0.3, 85.7]
Overexpression	32 (36)	19 (17)	2.8 [1.2, 12.7]
AOL [‡]			
0 compartments abnormal	18 (20)	34 (31)	1 (referent)
1 compartments abnormal	29 (32)	43 (39)	0.9 [0.3, 2.4]
2 compartments abnormal	28 (31)	25 (23)	1.7 [0.6, 5.1]
3 compartments abnormal	16 (18)	7 (6)	4.3 [0.7, 26.3]
Model performance [‡]			
C statistic			0.73

[†]Adjustments were made for current tobacco and alcohol use, BE segment length and time in follow-up;

[‡]Estimates are presented for the model with two groups of p53 expression.

Fig. 2 Receiver operating characteristic (ROC) curve for the multibiomarker panel.

Incorporating an expert LGD diagnosis as part of a prediction model for BE progression requires the availability of this service, a proposition that is unlikely in many parts of the world. Reported progression rates for confirmed LGD are highly divergent.^{26,37–40} This heterogeneity suggests that the diagnosis of LGD is markedly subjective in nature and that quality of the expert review will influence the predictive value. Furthermore, clinical management in BE patients with LGD is rapidly evolving. A high-quality expert consensus diagnosis of LGD has previously been demonstrated to be a strong predictor

of progression to HGD/EAC.^{26,38,39,41} Additionally, a recent randomized controlled trial demonstrated that prophylactic radiofrequency ablation (RFA) prevents neoplastic progression in BE patients with confirmed LGD.⁴² Current international guidelines, therefore, recommend to perform expert histological review in case of LGD and to consider patients for RFA treatment in case LGD is confirmed.^{3,7,9} The biomarkers described in this study have been shown to augment the effect of expert histological review. Especially in settings where histological review is suboptimal, these markers should be able to provide independent predictive power.

A limitation of the study was the exclusion of ploidy analysis from the biomarker panel as discussed above. A major strength of this study was the stringent selection of progressor patients, minimizing the possibility of prevalent HGD/EAC at baseline. Selection criteria included a minimum of 2 years of endoscopic surveillance before progression, an unequivocal diagnosis of the endpoint (HGD/EAC) with maximum stage T1 disease at time of progression. Assessment of all biomarkers was performed blinded to the outcome of the study. The community-based setting of our cohort as well as the collection of samples in routine clinical practice enhances the applicability of our results to community BE surveillance.

In conclusion, this nested case-control study validated findings from a previous study that identified a risk-stratification biomarker panel for BE.¹⁵ An expert consensus LGD diagnosis, abnormal expression of p53, and abnormal expression of AOL all independently predicted the risk of progression to HGD/EAC. The combination of these three markers can help select patients for prophylactic ablation therapy or intensified endoscopic surveillance.

Table 4 Sensitivity and specificity of the multibiomarker model at three cutpoints with 95% exact binomial confidence limits

	Cutpoints—percentile of the linear predictor		
	25	50	75
Sensitivity—Percent	88.9 [82.6, 95.6]	67.8 [58.8, 78.2]	41.1 [32.1, 52.6]
Specificity—Percent	35.8 [27.8, 46.0]	64.2 [55.8, 73.9]	88.1 [82.2, 94.4]

Table 5 Adjusted bivariate odds of neoplastic progression for the individual biomarkers and multibiomarker model performance in the current study compared with the previous study

Marker	Current study OR [95% CI]	Previous study (15) OR [95% CI]
Expert dysplasia		
No LGD	1 (referent)	1 (referent)
LGD	34.3 [3.4, 350.5]	11.8 [4.3, 32.2]
p53		
Negative	1 (referent)	1 (referent)
Abnormal	3.0 [1.1, 7.9]	1.6 [0.9, 2.8]
Cyclin A		
Negative	1 (referent)	1 (referent)
Positive	0.9 [0.2, 3.3]	1.3 [0.7, 2.7]
AOL		
0 compartments abnormal	1 (referent)	1 (referent) [†]
1 compartment abnormal	0.7 [0.3, 1.9]	—
2 compartments abnormal	1.3 [0.5, 3.4]	3.2 [1.7, 5.8]
3 compartments abnormal	4.1 [0.8, 20.6]	—
DNA copy number		
Diploid	—	1 (referent)
Abnormal	—	3.2 [1.7, 6.0]
Model performance		
C-statistic	0.73 [‡]	0.73 [§]

[†]Analyzed as 0–1 compartments abnormal versus 2–3 compartments abnormal;

[‡]Model includes expert dysplasia, p53 and AOL;

[§]Model includes expert dysplasia, DNA copy number and AOL.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Figure S1. Representative immunohistochemistry. Staining with antibodies for p53 (A and B), Cyclin A (C) and *Aspergillus Oryzae* lectin (AOL) (D). A: Aberrant p53 expression (absent staining pattern) on left, wild type expression on right; B: Aberrant p53 overexpression (strong staining); C: positive surface cells for Cyclin A; D: high intensity staining for AOL at the apical membrane, medium intensity staining for AOL of cytoplasmic mucin globule.

Supplementary Figure S2. Summary of image cytometry analysis. Representative examples of pictures of the whole nuclear field and histogram plots from a positive control (A), a successful run from a study sample (B), a study sample with too few cells (C), and whole nuclear field from a failed study sample (D). The whole nuclear field is a low power image of the well containing the nuclei. In the study samples, large debris can be seen and only a small number of nuclei are visible compared to the test sample. 100% of

test samples were viable for analysis. Out of 20 study samples tested, only 5 could be analyzed, 15 had too few cells for analysis. Number of events for test samples and study samples (E).

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