

A Multicenter, Double-Blinded Validation Study of Methylation Biomarkers for Progression Prediction in Barrett's Esophagus

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Abstract

Esophageal adenocarcinoma risk in Barrett's esophagus (BE) is increased 30- to 125-fold versus the general population. Among all BE patients, however, neoplastic progression occurs only once per 200 patient-years. Molecular biomarkers are therefore needed to risk-stratify patients for more efficient surveillance endoscopy and to improve the early detection of progression. We therefore performed a retrospective, multicenter, double-blinded validation study of eight BE progression prediction methylation biomarkers. Progression or nonprogression were determined at 2 years (tier 1) and 4 years (tier 2). Methylation was assayed in 145 nonprogressors and 50 progressors using real-time quantitative methylation-specific PCR. Progressors were significantly older than non-progressors (70.6 versus 62.5 years; $P < 0.001$). We evaluated a linear combination of the eight markers, using coefficients from a multivariate logistic regression analysis. Areas under the ROC curve (AUC) were high in the 2-, 4-year, and combined data models (0.843, 0.829, and 0.840; $P < 0.001$, <0.001 , and <0.001 , respectively). In addition, even after rigorous overfitting correction, the incremental AUCs contributed by panels based on the 8 markers plus age versus age alone were substantial (Δ -AUC = 0.152, 0.114, and 0.118, respectively) in all 3 models. A methylation biomarker-based panel to predict neoplastic progression in BE has potential clinical value in improving both the efficiency of surveillance endoscopy and the early detection of neoplasia. [Cancer Res 2009;69(10):OF1-4]

Introduction

Barrett's esophagus (BE) is a metaplastic condition where the normal squamous epithelium of the lower esophagus is replaced by a small intestinal-like columnar lining (1). Esophageal adenocarcinoma (EAC) risk in BE is increased 30- to 125-fold relative to the

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general population (2), and endoscopic surveillance in BE patients is recommended at intervals of 2 to 3 years (1, 3). EACs detected in surveillance programs occur at earlier stages and have better prognoses (4, 5), but endoscopic surveillance suffers from high cost, inconvenience, patient anxiety, low yield, and procedure-related risks. In addition, the current marker of EAC risk in BE, dysplasia, is plagued by high interobserver variability and limited predictive accuracy (6–8). Because neoplastic progression is infrequent in BE, the merits of and appropriate interval for endoscopic surveillance in BE have led to frequent debate (3, 5). This process would benefit greatly from effective biomarkers to stratify patients according to their level of neoplastic progression risk.

In 2005, we reported that hypermethylation of *p16*, *RUNX3*, and *HPPI* occurs early in BE-associated neoplastic progression and predicts progression risk (9). Later, we developed a tiered risk stratification model to predict progression in BE using epigenetic and clinical features (10). We also studied methylation levels and frequencies of individual genes using real-time quantitative methylation-specific PCR in 259 endoscopic esophageal biopsy specimens of differing histologies. Among 10 genes evaluated, five, namely *nel-like 1* (*NELL1*), *tachykinin-1* (*TAC1*), *somatostatin* (*SST*), *AKAP12*, and *CDH13*, were methylated early and often in BE-associated neoplastic progression (11–15). In the above studies, methylation status and levels correlated inversely with mRNA expression levels (9–15). In light of these findings, we performed a retrospective, multicenter, double-blinded validation study of these eight methylation biomarkers (i.e., *p16*, *RUNX3*, *HPPI*, *NELL1*, *TAC1*, *SST*, *AKAP12*, and *CDH13*) for their accuracy in predicting neoplastic progression in BE.

Materials and Methods

Definition of BE progressor and nonprogressor patients and sample collection. Progressors and nonprogressors were defined as described previously (10). Progressors were considered both as a single combined group, and in 2 tiers: progression within 2 y (tier 1) or 4 y (tier 2). One hundred ninety-five BE biopsies (145 nonprogressors and 50 progressors) were obtained from 5 participating centers: the Mayo Clinic at Rochester/Jacksonville, the University of Arizona, the University of North Carolina, and Johns Hopkins University. All patients provided written informed consent under a protocol approved by Institutional Review Boards at their institutions. Biopsies were taken using a standardized biopsy protocol (9, 10). Clinicopathologic features are summarized in Supplementary Table S1.

Table 1. Logistic regression and overfitting correction for the 2-y, 4-y, and combined models

	AUC1 (95% CI)	P of AUC1	AUC2 (95% CI)	P of AUC2	Shrinkage (AUC1-AUC2)
2-y model	0.843 (0.763, 0.924)	<0.001	0.745 (0.685, 0.875)	0.001	0.098
4-y model	0.829 (0.773, 0.907)	<0.001	0.720 (0.694, 0.856)	0.004	0.109
Combined model	0.840 (0.773, 0.907)	<0.001	0.732 (0.697, 0.868)	0.002	0.108

Abbreviations: AUC1, original AUC; AUC2, overfitting corrected AUC; CI, confidence interval.

Bisulfite treatment and real-time quantitative methylation-specific PCR. Bisulfite treatment was performed as described (11). Promoter methylation levels of eight genes (*p16*, *HPPI*, *RUNX3*, *CDH13*, *TAC1*, *NELLI*, *AKAP12*, and *SST*) were determined by quantitative methylation-specific PCR on an ABI 7900 Sequence Detection (Taqman) System (11). β -*actin* was used for normalization. Primers and probes for quantitative methylation-specific PCR are described in Supplementary Table S2. A standard curve was generated using serial dilutions of CpGenome Universal Methylated DNA (CHEMICON). A normalized methylation value for each gene of interest was defined as described (11). Wetlab analysts (ZJ and YC) and all SJM laboratory personnel were blinded to specimen progressor or nonprogressor status.

Data analysis and statistics. Associations between progression status and patient characteristics were tested using Student's *t* test or χ^2 testing. Relationships between biomarkers and patient progression status were examined using Wilcoxon rank-sum testing.

To evaluate the predictive utility of the markers, we constructed receiver operating characteristic (ROC) curves. ROC curve analyses were first conducted on individual markers, then in combination to determine whether a panel performed better than any single marker. Our algorithm rendered a single composite score, using the linear predictor from a binary regression model justified under the linearity assumption (16). The predictive accuracy of composite scores was evaluated based on a resampling algorithm: we randomly split data into a learning set containing two of three and a test set including one of three of observations. The combination rule derived from the learning set produced two ROC curves, from the learning and test sets, respectively. Vertical differences between these two ROC curves yielded the overestimation of sensitivities at given specificities. This procedure was repeated 200 times, and these 200 differences were averaged to estimate the expected overfitting.

We also used predictiveness curves (17) to display risk distribution as a function of the combined marker in the population. This curve represents a plot of risk associated with the ν^{th} quantile of the marker, $P\{D = 1|Y = F^{-1}(\nu)\}$ versus ν , with $F(\cdot)$ the cumulative distribution of the marker. These plots display population proportions at different risk levels more clearly than do other metrics (such as ROC curves). Because a case-control sample was studied, we used an external progression prevalence rate to calculate risk in the targeted screening population. To calibrate for future samples, a shrinkage coefficient estimated from the logistic regression model was applied to the linear predictors from which risk was calculated (18).

All analyses were performed in R.¹⁰ Statistical data analysts (Y.Z., W.G., and Z.F.) were blinded to the identities of the eight biomarkers.

Results

Clinical characteristics. Progressors versus nonprogressors did not differ significantly by gender; body mass index; BE segment length; LGD patient percentage; family history of BE, LGD, HGD, or EAC; cigarette smoking; or alcohol use; however, progressors were

significantly older than nonprogressors (70.6 versus 62.5 years; $P < 0.001$, Student's *t* test; Supplementary Table S1). Samples consisted of one biopsy from each of 50 progressors and 145 nonprogressors (195 patients) in the combined model. In the 2-year model, we redefined progressors whose interval from index to final procedure exceeded 2 years as nonprogressors, yielding 36 progressors and 159 nonprogressors. In the 4-year model, we redefined progressors whose interval from index to final procedure exceeded 4 years as nonprogressors, yielding 47 progressors and 148 nonprogressors.

Univariate analyses. Normalized methylation values of *HPPI*, *p16*, and *RUNX3* were significantly higher in progressors versus nonprogressors by Wilcoxon test (0.456, 0.138, and 0.104 versus 0.273, 0.069, and 0.063; $P = 0.0025$, 0.0066, and 0.0002, respectively). The remaining five markers did not differ significantly in progressors versus nonprogressors (Supplementary Table S3). We further assessed the classification accuracy of single markers using ROC curve analyses. Areas under ROC curve (AUC) for *HPPI*, *p16*, and *RUNX3* were all significantly >0.50 (Supplementary Table S4).

Logistic regression analyses of the eight-marker panel. We then combined all eight markers by performing logistic regression and treating them as linear predictors (Supplementary Fig. S1; Table 1). All models exhibited high AUCs (0.843, 0.829, and 0.840, respectively; Supplementary Fig. S1A–C; Table 1). We performed overfitting correction based on 3-fold cross-validation and 200 bootstraps. The overfitting-corrected AUCs remained high (0.745, 0.720, and 0.732, respectively), whereas shrinkages from overfitting correction were modest (0.098, 0.109, and 0.108, respectively) in the 3 models (Supplementary Fig. S1A–C; Table 1).

We also explored the incremental AUC value contributed by an eight-marker-plus-age panel to that of age alone (Supplementary Fig. S1; Table 2). The AUCs of the 8-marker-plus-age panels in the 3 models (0.858, 0.850, and 0.855, respectively) were higher than those of age alone (0.604, 0.630, and 0.635, respectively; Supplementary Fig. S1D–F; Table 2). Overfitting-corrected AUCs remained high (0.756, 0.744, and 0.753, respectively), and increments contributed by the age-plus-biomarker panel versus age were substantial (0.152, 0.114, and 0.118, respectively) in the 3 models (Supplementary Fig. S1D–F; Table 2).

Sensitivity and specificity of the eight-marker panel. Although maintaining high specificity to minimize false-positive results, our model still predicted a number of new early diagnoses, i.e., diagnoses that would not have occurred earlier without the panel (Table 3). Although maintaining specificity at 0.9 or 0.8, sensitivities (0.443 and 0.629 for the combined model, 0.607 and 0.721 for the 2-year model, and 0.465 and 0.606 for the 4-year model, respectively) were above or approached 50% in all three models based on the 8-marker panel alone. Furthermore, at 0.9 or 0.8 specificities, sensitivities (0.457 and 0.757 for the combined

¹⁰ <http://www.r-project.org>

Table 2. Incremental values above age alone for the 2-y, 4-y, and combined models

	AUC1 (95%CI)	AUC2 (95%CI)	AUC3 (95%CI)	Increment over age (AUC3-AUC1)
2-y model	0.604 (0.491, 0.718)	0.858 (0.783, 0.932)	0.756 (0.699, 0.884)	0.152
4-y model	0.630 (0.526, 0.735)	0.850 (0.784, 0.917)	0.744 (0.719, 0.871)	0.114
Combined model	0.635 (0.532, 0.737)	0.855 (0.790, 0.919)	0.753 (0.729, 0.878)	0.118

NOTE: AUC, area under the receiver-operator characteristic curve; AUC1, AUC of age alone; AUC2, AUC of age plus markers; AUC3, overfitting corrected AUC of age plus markers.

model, 0.536 and 0.786 for the 2-year model, and 0.450 and 0.724 for the 4-year model, respectively) exceeded or approached 50% in all models based on the 8-marker-plus-age panel.

Risk stratification of BE patients. ROC curves derived from these marker-based models were used to establish thresholds to stratify patients into risk categories. This procedure was performed to identify high-risk individuals for more frequent endoscopic screening. The threshold above which patients were classified as high-risk was chosen at *specificity* = 90%, to minimize false-positive, unnecessary endoscopies (type II error). A second threshold was established to identify low-risk individuals for less frequent endoscopic screening. The threshold below which patients were classified as low-risk was chosen at a sensitivity of 90%, to minimize false-negative, missed high-risk individuals (type I error). Based on the combined progressor and nonprogressor classification, we classified patients as low risk with a threshold that corresponded to 90% true-positives and 43% false-positives; the high-risk group was defined using a threshold that yielded 43% true-positives and 10% false-positives. Assuming a cumulative progression rate to HGD and/or EAC of 7.5% over 5 years (19), the corresponding negative predictive value relating to our low-risk threshold was 98.7% (i.e., progression risk in the low-risk group was 1.3%) and the positive predictive value relating to high risk was 27% (i.e., progression risk in the high-risk group was 27%).

Predictiveness curve analyses. We used predictiveness curves (also known as risk plots) to assess the clinical utility of the combined classification rules in stratifying patients according to risk levels in the target population. To create predictiveness curves,

we ordered and plotted risks from lowest to highest value. A progression rate to HGD and/or EAC of 7.5% over 5 years (19) was assumed in adjusting estimates from the case-control sample to reflect population risk and its distribution. Results are shown in Table 4 and Supplementary Fig. S2. After overfitting correction, by age alone, nearly 90% of BE patients were classified as intermediate-risk, whereas patients were well-stratified into low-risk, intermediate-risk, or high-risk categories by both the 8-marker alone and age plus 8-marker panels in all three models (Supplementary Fig. S2; Table 4).

Discussion

In the current study, with specificity at 0.9, sensitivities of progression prediction approached 50% based on both the 8-marker panel alone and 8-marker-plus-age panel in all three models. These findings indicate that although performing at high specificity, these biomarker models predicted half of progressors to HGD and EAC that would not have been diagnosed earlier without using these biomarkers.

Based on age alone, with specificity at 90%, only 17.6%, 23.2%, and 22.1% of progressors were predicted in the three models. However, with panels based on age plus biomarkers or on biomarkers alone, ~60%, 50%, and 50% of progressors were accurately predicted in these 3 models. Predicted progressors represent patients in whom we can intercede earlier, resulting in higher cure rates. Finally, our combined risk model outperformed known risk in the general BE population (7.5% progression risk over 5 years), both in terms of

Table 3. Specificity and sensitivity for 2-y, 4-y, and combined models

	Specificity (95% CI) at sensitivity		Sensitivity (95% CI) at specificity	
	0.9	0.8	0.9	0.8
Combined model				
Age	0.260 (0.162, 0.425)	0.390 (0.240, 0.508)	0.221 (0.054, 0.448)	0.371 (0.204, 0.532)
Marker combination	0.567 (0.413, 0.849)	0.724 (0.574, 0.914)	0.443 (0.350, 0.838)	0.629 (0.527, 0.941)
Age+marker combination	0.576 (0.484, 0.867)	0.781 (0.647, 0.944)	0.457 (0.372, 0.869)	0.757 (0.598, 0.964)
2-y model				
Age	0.205 (0.138, 0.389)	0.351 (0.197, 0.484)	0.176 (0.021, 0.426)	0.354 (0.172, 0.538)
Marker combination	0.547 (0.436, 0.873)	0.757 (0.595, 0.935)	0.607 (0.393, 0.870)	0.721 (0.593, 0.969)
Age+marker combination	0.615 (0.474, 0.918)	0.786 (0.652, 0.956)	0.536 (0.400, 0.934)	0.786 (0.600, 0.987)
4-y model				
Age	0.249 (0.158, 0.430)	0.384 (0.229, 0.506)	0.232 (0.038, 0.467)	0.382 (0.214, 0.541)
Marker combination	0.523 (0.426, 0.835)	0.704 (0.579, 0.909)	0.465 (0.346, 0.814)	0.606 (0.545, 0.941)
Age+marker combination	0.574 (0.488, 0.864)	0.757 (0.649, 0.940)	0.450 (0.385, 0.885)	0.724 (0.600, 0.963)

Table 4. Overfitting-corrected predictiveness curve analyses in 2-yr, 4-y, and combined models

	Risk probability		
	<0.1 (LR)	0.1–0.5 (IR)	>0.5 (HR)
Combined model			
8-marker panel	45%	51%	4%
Age alone	15%	85%	0%
Age plus 8-marker panel	51%	44%	5%
2-y model			
8-marker panel	45%	51%	4%
Age alone	11%	89%	0%
Age plus 8-marker panel	52%	44%	4%
4-y model			
8-marker panel	44%	51%	5%
Age alone	15%	85%	0%
Age plus 8-marker panel	51%	44%	5%

NOTE: LR, low risk; IR, intermediate risk; HR, high risk.

negative predictive value (1.3% progression risk over 5 years for the low-risk group) and positive predictive value (27% progression risk over 5 years for the high-risk group).

Age is a common risk factor for many cancers, including EAC (20). In the current study, progressors were significantly older than nonprogressors, and the AUCs of age alone were 0.604, 0.630, and 0.635, respectively in the 3 models, suggesting that age per se predicts neoplastic progression in BE. However, methylation of tissues increases with aging, even in the absence of neoplastic progression (21, 22). Thus, aging may exert risk on progression either independently, or through its influence on methylation. Nevertheless, the incremental prediction accuracy (above age) contributed by the eight-marker panel was substantial in all three models.

Thus, the current findings suggest that this eight-marker panel is more objective and quantifiable and possesses higher predictive sensitivity and specificity than do clinical features, including age.

Furthermore, although age was a good classifier for disease progression, predictiveness curves revealed that age did not successfully stratify BE patients according to their progression risk. Moreover, age per se is not an accepted risk marker on which to base clinical decisions regarding surveillance interval or neoplastic progression risk in BE. In contrast, models based on both the 8-marker panel and the age-plus-8-marker panel provided estimated progression risks either close to 0 (i.e., low risk) or between 0.1 and 0.5 (i.e., intermediate risk) in the majority of individuals, suggesting that these markers exerted a substantial effect on risk category. This finding also suggests that in clinical practice, separate thresholds can be chosen to define high, intermediate, and low risk, based on predictiveness curves.

In conclusion, we have developed a risk stratification strategy to predict neoplastic progression in BE patients based on an eight-marker tissue methylation panel. At high specificity levels, this model accurately predicted approximately half of HGDs and EACs that would not have otherwise been predicted. This model is expected to reduce endoscopic procedures performed in BE surveillance while simultaneously increasing detection at earlier stages. Future studies should explore additional potentially predictive methylation targets, along with alternative means of assessing methylation biomarkers (such as immunohistochemical staining for reduced biomarker expression). Thus, these findings suggest that a methylation biomarker panel offers promise as a clinically useful tool in the risk stratification of BE patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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