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Derivation of genetic biomarkers for cancer risk stratification in Barrett's oesophagus: a prospective cohort study

Margriet R. Timmer^{1,*}, Pierre Martinez^{2,*}, Chiu T. Lau¹, Wytske M. Westra¹, Silvia Calpe¹, Agnieszka M. Rygiel¹, Wilda D. Rosmolen¹, Sybren L. Meijer³, Fiebo J.W. ten Kate³, Marcel G.W. Dijkgraaf⁴, Rosalie C. Mallant-Hent⁵, Anton H.J. Naber⁶, Arnoud H.A.M. van Oijen⁷, Lubbertus C. Baak⁸, Pieter Scholten⁹, Clarisse J.M. Böhmer¹⁰, Paul Fockens¹, Carlo C. Maley¹¹, Trevor A. Graham², Jacques J.G.H.M. Bergman¹, and Kausilia K. Krishnadath¹

¹ Department of Gastroenterology and Hepatology, Academic Medical Centre – University of Amsterdam, 1011 AZ, Amsterdam, The Netherlands ² Evolution and Cancer Laboratory, Centre for Tumour Biology, Barts Cancer Institute, London, EC1M 6BQ, United Kingdom ³ Department of Pathology, Academic Medical Centre – University of Amsterdam, 1011 AZ, Amsterdam, The Netherlands ⁴ Clinical Research Unit, Academic Medical Centre – University of Amsterdam, 1011 AZ, Amsterdam, The Netherlands ⁵ Department of Gastroenterology, Flevoziekenhuis, 1315 RA, Almere, The Netherlands ⁶ Department of Gastroenterology, Tergooiziekenhuizen, 1213 XZ, Hilversum, The Netherlands ⁷ Department of Gastroenterology, Medisch Centrum Alkmaar, 1815 JD The Netherlands ⁸ Department of Gastroenterology, Onze Lieve Vrouwe Gasthuis, 1091 AC, Amsterdam, The Netherlands ⁹ Department of Gastroenterology, Sint Lucas Andreas Ziekenhuis, 1061 AE, Amsterdam, The Netherlands ¹⁰ Department of Gastroenterology, Spaarne Ziekenhuis, 2134 TM, Hoofddorp, The Netherlands ¹¹ Centre for Evolution and Cancer, University of California at San Francisco, CA 94143-0128, USA

Abstract

Correspondence: Kausilia K. Krishnadath, MD PhD, Professor of Translational Gastroenterology, Department of Gastroenterology and Hepatology, Academic Medical Centre Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands, k.k.krishnadath@amc.uva.nl, Phone number: +31-205666703, Fax: +31-206917033.
*joint first authors

Contributors:

KKK designed the study. MRT, CTL, WMW, AMR, WDR, SLM, FJWtK, RCM-H, AHJN, AHAMvO, LCB, PS, CJMB, PF, JJGHMB, and KKK did the data collection. MRT, PM, SC, MGWD, TAG, CCM, and KKK did the statistical data analysis and interpretation of statistical data; MRT, PM, TAG, CCM, and KKK wrote the paper and created the figures. All authors reviewed the drafts of the paper and gave final approval of the version to be published.

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Objective—The risk of developing adenocarcinoma in non-dysplastic Barrett's oesophagus is low and difficult to predict. Accurate tools for risk stratification are needed to increase the efficiency of surveillance. We aimed to develop a prediction model for progression using clinical variables and genetic markers.

Methods—In a prospective cohort of patients with non-dysplastic Barrett's oesophagus, we evaluated six molecular markers: *p16*, *p53*, *Her-2/neu*, *20q*, *MYC*, and aneusomy by DNA fluorescence in situ hybridisation on brush cytology specimens. Primary study outcomes were the development of high-grade dysplasia or oesophageal adenocarcinoma. The most predictive clinical variables and markers were determined using Cox proportional-hazards models, receiver-operating-characteristic curves and a leave-one-out analysis.

Results—A total of 428 patients participated (345 men; median age 60 years) with a cumulative follow-up of 2019 patient-years (median 45 months per patient). Of these patients, 22 progressed; nine developed high-grade dysplasia and 13 oesophageal adenocarcinoma. The clinical variables, age and circumferential Barrett's length, and the markers, *p16* loss, *MYC* gain, and aneusomy, were significantly associated with progression on univariate analysis. We defined an 'Abnormal Marker Count' that counted abnormalities in *p16*, *MYC* and aneusomy, which significantly improved risk prediction beyond using just age and Barrett's length. In multivariate analysis, these three factors identified a high-risk group with an 8.7-fold (95% CI, 2.6 to 29.8) increased hazard ratio compared with the low-risk group, with an area under the curve of 0.76 (95% CI, 0.66 to 0.86).

Conclusion—A prediction model based on age, Barrett's length, and the markers *p16*, *MYC*, and aneusomy determines progression risk in non-dysplastic Barrett's oesophagus.

Keywords

Barrett Oesophagus; Biological Markers; Oesophageal Adenocarcinoma; Endoscopic Surveillance; Cytology

INTRODUCTION

Barrett's oesophagus is a premalignant condition of the lower oesophagus in which, as a result of longstanding reflux disease, the normal squamous mucosa is replaced by metaplastic columnar epithelium.[1] Barrett's oesophagus may progress to oesophageal adenocarcinoma, a cancer with poor prognosis unless detected at early stage.[2]

Most Barrett's oesophagus cases are diagnosed as having no dysplasia and the risk of malignant progression in non-dysplastic Barrett's oesophagus is remarkably low with annual rates of 0.12%-0.6%.[3,4] The presence of dysplasia on endoscopic biopsies is currently the only widely used biomarker for risk stratification. Diagnosing and grading of dysplasia are, however, subject to considerable interobserver and intraobserver variation and require assessment by expert pathologists.[5] Current guidelines recommend endoscopic surveillance with biopsies to detect dysplasia and early malignancies. High-grade dysplasia and early-stage cancers both can be treated effectively by endoscopic intervention with comparable outcomes.[2,6] Due to the low progression rate of non-dysplastic Barrett's

oesophagus, surveillance represents a labour-intensive practice of highly questionable cost-effectiveness.[7]

Therefore, it is of paramount importance to increase the efficiency of surveillance via the development of innovative measures that can identify patients at high risk of progression who may require early intervention, or patients with a minimal progression risk in whom the frequency of surveillance can be tempered. It has been recently shown that 50% of the non-dysplastic Barrett's oesophagus cases contain genetic abnormalities that may reveal the first steps in the progression to cancer.[8] Because these abnormalities occur much earlier than overt histological changes, they may represent an invaluable source of potential biomarkers.

Several genetic abnormalities have been studied to identify biomarkers that are associated with progression. Abnormalities of the tumour suppressor genes *p16* and *p53* and genetic instability as measured by DNA content abnormalities (ie, ploidy changes) are the most extensively studied.[9–12] These abnormalities may arise early on and can expand in large areas within the Barrett's epithelium.[13] In a prospective cohort study (n=243), a biomarker panel including 17p LOH (*p53*), abnormal DNA ploidy status, and 9p LOH (*p16*) predicted progression to oesophageal adenocarcinoma with a relative risk of 38.7 (95% CI, 10.8 to 138.5).[12] In a case-control study, a combination of low-grade dysplasia, abnormal DNA ploidy, and *Aspergillus oryzae* lectin identified progressors and non-progressors.[10] Other genetic abnormalities with potential prognostic significance are amplification of the oncogenes *Her-2/neu* and *MYC*. [14,15] Using single nucleotide polymorphism arrays to assay copy-number changes, genome-wide, a recent case-cohort study demonstrated a higher genetic diversity in progressors compared with non-progressors.[16] Progressors had multiple losses, gains and genome doublings detectable up to 48 months before oesophageal adenocarcinoma occurred, suggesting a window of opportunity for early recognition of progressors.

Nevertheless, prospective studies evaluating the performance of biomarkers in non-dysplastic Barrett's surveillance cohorts are lacking. In cross-sectional studies, we and others demonstrated that the most frequently observed genetic abnormalities in Barrett's oesophagus, including *p16* and *p53* loss, aneusomy and amplifications of *MYC* and *Her-2/neu*, are efficiently detected by DNA fluorescence in situ hybridisation (FISH), a technique that has the advantage that it can be applied on brush cytology specimens obtained from the entire Barrett's epithelial surface as compared with random biopsies. Therefore, it has the potential to decrease sampling error and moreover it allows an evaluation of genetic abnormalities in a quantitative fashion that reflects the extent of mutant field defects within the Barrett's segment. In addition, several types of genetic abnormalities, for example, gene losses or gains and aneusomy at multiple loci can be detected by FISH in a single assay. [15,17] Here, we evaluated six candidate biomarkers using DNA FISH in a large prospective cohort, and designed a prediction model based on the most predictive clinical variables and genetic markers.

METHODS

Study design and patients

The study included patients from six general hospitals and one academic medical centre in the Amsterdam region of the Netherlands between 2002 and 2013. Only patients with non-dysplastic Barrett's oesophagus with a maximum Barrett's segment length of 1 cm were enrolled.[18] Barrett's oesophagus was defined as the presence of columnar-lined epithelium in the distal oesophagus, visible as pink mucosa extending above the top of the gastric folds during endoscopy, and confirmed by the presence of specialised intestinal metaplasia in biopsies.[19] Exclusion criteria were (a) age <18 years or >80 years, (b) the presence of low or high-grade dysplasia or adenocarcinoma at the index endoscopy, (c) a history of high-grade dysplasia or oesophageal adenocarcinoma, (d) prior endoscopic therapy for Barrett's oesophagus, (e) active reflux disease, and (f) the lack of an evaluable brush cytology specimen. Primary study outcomes were the development of high-grade dysplasia or oesophageal adenocarcinoma. Patients with histological progression within 6 months were excluded from the final analysis since dysplasia or adenocarcinoma may have been missed initially due to biopsy sampling inadequacy. All patients included in the final analysis underwent at least one follow-up endoscopy. Follow-up time was measured from the date of the index endoscopy to the date of progression or to the date of the last follow-up endoscopy for those who did not progress.

Endoscopic surveillance and prospective registration

All patients participated in a prospective surveillance programme. Prospective registration was coordinated by two experienced research nurses with a minimum of 3-year experience in Barrett's oesophagus. All endoscopies were performed with strict adherence to current surveillance guidelines.[2] Patients with no dysplasia underwent surveillance endoscopy every 2-3 years, while those with a first diagnosis of Barrett's oesophagus also underwent repeat endoscopy within 6 months.[20] During the index endoscopy and each follow-up endoscopy, biopsies were taken according to the Seattle protocol (four-quadrant biopsies/2-cm intervals) and from any visible lesions. All surveillance endoscopies in the seven participating hospitals were attended by one of the research nurses to collect clinical data and brush cytology material that was obtained during the index endoscopy. Endoscopic details and corresponding histological results were recorded in a prospectively maintained database.

Histopathology review

Formalin-fixed, paraffin-embedded biopsies were cut into 5 µm-thick sections, stained with H&E and evaluated for histopathology. Biopsies were assessed for the presence of intestinal metaplasia, dysplasia and adenocarcinoma according to the Vienna classification by the local pathologists from the seven centres. Following current guidelines, all dysplasia and adenocarcinoma cases were reviewed by a panel of expert pathologists.[19] All pathologists were blinded to the outcome of the biomarker analysis.

Brush cytology and FISH

Cytology specimens were collected at the index endoscopy by sampling the entire Barrett's segment with a standard cytology brush (Cook Endoscopy, Winston-Salem, North Carolina, USA). Prior to brushing, the Barrett's mucosa was sprayed with acetylcysteine (50mg/mL) to dissolve the mucus layer. Cells were released from the brush by rigorous shaking of the brush in a 20mL vial with PreservCyt solution (Hologic, Marlborough, Massachusetts, USA) and concentrated in 3 mL by removal of the supernatant. The cytopspin procedure (Shandon Cytospin 4, Cytocentrifuge, Thermo, Waltham, Massachusetts, USA) was performed as previously described to concentrate the cells on a slide in a uniform monolayer after which the slides were stored at -80°C . [15]

We performed genetic analysis by DNA FISH as previously described using locus-specific probes to *p16* (CDKN2A), *p53* (TP53), *Her-2/neu* (ERBB2), *20q*, and *MYC*, and centromeric probes for chromosomes 7 and 17 (Abbott Molecular) to detect aneusomy. [15] Probe signals were visualised using a fluorescent microscope (Olympus BX61) equipped with specific band filters for each of the probes. Slides were scored by two experienced technicians by recording signal patterns in 100 (minimum 75) consecutive interphase nuclei of non-squamous, non-inflammatory cells. Data were described as the percentage of cells showing genetic abnormalities. Previously, we showed that FISH for two centromeric probes correlated with DNA image cytometry analysis to detect DNA content abnormalities in Barrett's oesophagus. Therefore, aneusomy detected by the centromeric probes for CEP7 and CEP17 was used as a surrogate marker to assess DNA ploidy changes. If abnormalities (both losses and gains) were observed for both CEP7 and CEP17, we used the mean percentage of abnormal cells for both probes as a continuous variable. All FISH analyses were performed prospectively and blinded for patient outcomes.

Defining clinical and molecular variables

Clinical risk factors (clinical variables) evaluated in the study were age, sex, body mass index, tobacco use, family history of Barrett's or oesophageal adenocarcinoma, and circumferential Barrett's segment length and the molecular markers were *p16*, *p53*, *her-2/neu*, *20q*, *MYC*, and aneusomy. To define the best descriptors for the variables, each variable was described either as a 'continuous' or 'binary' variable. The threshold for binarisation was chosen as follows: the goodness of a particular descriptor was defined using a bootstrap approach that involved splitting the cohort into two, with equal numbers of progressors in each half, and then evaluating the ability of a Cox model fitted to the training set to predict progression risk on the test set, using the area under the curve (AUC) as the performance measure. The best descriptor of each variable was defined as the one yielding the highest median AUC across the bootstrap replicates. Then, we performed univariate Cox proportional-hazards analyses to select variables that were significantly associated with progression. In addition, we created a variable named 'Abnormal Marker Count' that counted the number of abnormal markers among the markers that were significantly associated with progression on univariate analysis (ie, *p16* loss, *MYC* gain and aneusomy). For the Abnormal Marker Count the optimal thresholds for binary stratification were used for each marker, while each individual marker was scored on a continuous scale.

Development of a risk model

We developed a series of multivariate Cox proportional-hazards models, that included various combinations of the molecular markers and clinical variables that were significant in the univariate analysis, and compared their predictive value over-and-above a model containing only clinical variables. Model performance was tested using a training/test set bootstrap approach and evaluation of both the AUC and Akaike's Index Criterion (AIC) to prevent over-fitting.[21,22] The AUC was computed as a measure of discrimination and provides information about the ability of a model to discriminate between different outcomes (progressors *vs* non-progressors), whereas the AIC is a measurement of information loss which takes into account both the goodness of fit and the complexity of the model, by including a penalty for additional parameters in a model.

The models tested were: (1) the significant clinical variables on univariate analysis (*Clinical model*), (2) a model comprising the significant clinical variables and all molecular markers (*Clinical + All markers*), (3) a set of models with the significant clinical variables in combination with each individual marker that was significant on univariate analysis (*Clinical + molecular models*), (4) the significant clinical variables and all significant markers (*Clinical + Significant molecular markers*), and (5) the significant clinical variables and the number of abnormal markers that were significant on univariate analysis (*p16 loss, MYC, and/or aneusomy*) that a patient had at baseline (*Clinical + Abnormal Marker Count*).

Next, for the best prediction models, risk scores were calculated for each patient. The risk score was defined as the relative risk of progression of a patient compared with the mean of the cohort:

$$R = \frac{h(t)}{\bar{h}(t)} = \frac{\exp(\sum_i \beta_i x_i)}{\exp(\sum_i \beta_i \bar{x}_i)}$$

where the x_i denotes the value of the i^{th} variable, variable, β_i the coefficients derived from the multivariate model, and $\bar{x}_i$ the average value of each variable. These calculations were performed using the predict.coxph function from the “survival” package in R. A leave-one-out analysis was used to test the robustness of the predictive value of the risk scores for each model. The multivariate model yielding the highest sensitivity in the leave-one-out analysis was selected as the best multivariate model. Kaplan-Meier curves were generated with the survplot R package.[23] P values < 0.05 were considered significant. Further details are included in the online supplementary methods.

RESULTS

Patients

Of the initial 674 patients who were enrolled, 498 met the inclusion criteria (see online supplementary figure S1). The cumulative follow-up time of the whole population was 2277 patient-years, and the median follow-up time per patient was 43 months (IQR, 36-72).

During follow-up, 22 patients progressed after a median of 34 months (IQR, 24-38 months); nine patients developed high-grade dysplasia and 13 patients progressed to oesophageal adenocarcinoma. Three of the patients who developed high-grade dysplasia had an intermediate diagnosis of low-grade dysplasia. The rate of progression was 0.97% (95% CI 0.61 to 1.46) per patient-year for the combined endpoint of high-grade dysplasia and adenocarcinoma and 0.57% (95% CI 0.30 to 0.97) for adenocarcinoma alone.

Evaluable brushes were obtained from 428 participants, which included all 22 progressors. DNA FISH was performed for the evaluation of genetic abnormalities (see online supplementary figures S2 and S3). The cumulative follow-up period of the participant group was 2019 patient-years and the median follow-up per patient was 45 months (IQR 35–72 months). Patient characteristics of patients included and not included in the final analysis are shown in table 1 and online supplementary table S1. The frequencies of genetic abnormalities at baseline are summarised in figure 1.

Prediction of progression

The best descriptors of each variable were defined by a bootstrap-based analysis (see online supplementary figure S4, for a detailed explanation see the Method section). Of the clinical variables, age and the circumferential Barrett's segment length were significant predictors of progression, while *p16* loss, *MYC* gain, aneusomy and the Abnormal Marker Count were the molecular markers significantly associated with progression (table 2).

Following univariate analysis, a series of different multivariate prediction models were compared. The properties of each model are reported in online supplementary table S2 and figure 2. Of all models that had both lower AIC and higher AUC than the *Clinical model*, the *Clinical + Significant molecular markers model* corresponded to the best sensitivity and negative predictive value, as given by the highest median AUC, while the *Clinical + Abnormal Marker Count* model corresponded to the lowest median AIC, indicating good prediction with low information loss.

The final model was selected on the basis of predictive power and robustness as determined by a risk-score-based leave-one-out analysis. In this analysis, the *Clinical + Abnormal Marker Count* model, which included age, Barrett's segment length and the 'Abnormal Marker Count' that indicated how many of the three significant molecular markers were abnormal for each patient, was the most significant predictor, achieving a sensitivity of 0.86 and a specificity of 0.54 (see online supplementary figure S5). In this model, the Marker Count proved to be the most significant predictor of progression (HR, 1.91, 95% CI 1.27 to 2.76, $p=0.002$; table 3) and reduced the risk of over-fitting. The addition of the Abnormal Marker Count improved both the AUC and the AIC compared with the *Clinical model* (0.76 (95% CI 0.66 to 0.86] vs 0.69 (95% CI 0.60 to 0.80] and 104.0 (95% CI 93.5 to 113.3] vs 107.1 (95% CI 99.3 to 114.2), respectively; both $p<2.2e^{-16}$, paired t-tests). Circumferential Barrett's length was the weakest predictor in the model, but contributed significantly to the overall model in terms of sensitivity, as a model comprising only age and the Abnormal Marker Count achieved lower sensitivity (see online supplementary figure S6). The *Clinical + Significant molecular markers* model achieved similar specificity of 0.54, but lower sensitivity of 0.73 and was likely prone to over-fitting (see online supplementary figure S5).

The *Clinical + Abnormal Marker Count* model was therefore used to classify patients as low and high-risk. Patients were high-risk if their risk score was greater than “one” as higher thresholds would hamper sensitivity (see online supplementary table S3). Overall, patients in the low-risk group (n=221) had significantly better progression-free survival than patients in the high-risk group (n=207; HR=8.7, 95% CI 2.6 to 29.8; log-rank $p<0.001$; figure 3). Progression-free survival at 5-years of follow-up was 98.6% for the low-risk group and 92.8% for the high-risk group. Of the 22 progressors, 19 were designated as ‘high-risk’ and three as ‘low-risk’. The three ‘misclassified’ patients progressed after 14, 24, and 34 months, respectively. Two of these patients had an Abnormal Marker Count of ‘0’ and the remaining patient had an Abnormal Marker Count of “one” but only had tongues of Barrett’s mucosa and no circumferential Barrett’s segment. The specificity of the model was 0.54 meaning that 46% of the non-progressors would be incorrectly classified as high-risk. The positive predictive value of the model was low at 9%, but its negative predictive value was 99%, meaning that 99% of the patients would be safely classified as low-risk and not progress to high-grade dysplasia or cancer during the studied time of follow-up.

DISCUSSION

Numerous studies have evaluated predictive biomarkers in Barrett’s oesophagus, but there have been no large prospective studies that focused solely on non-dysplastic Barrett’s patients. These patients represent over 95% of the Barrett’s surveillance population and have an annual progression risk as low as 0.12%-0.6%. [3,4] Surveillance of this difficult patient group is therefore questionable and identifying robust stratifying biomarkers to improve cost-effectiveness of surveillance is highly desirable. [7] We note that previously published cohort biomarker studies frequently included both non-dysplastic Barrett’s oesophagus patients and patients with low-grade dysplasia. [12,24] Manifest low-grade dysplasia may increase the annual progression risk approximately 50-fold to 100-fold to 13%. [5] This means that outcomes of studies in mixed cohorts have been largely affected by the outcomes of patients with low-grade dysplasia at baseline.

Here, we have presented the largest study to date in which we evaluated six putative genetic markers in combination with clinical variables for determining progression risk in a prospective cohort of patients with non-dysplastic Barrett’s oesophagus. The evaluation of genetic markers in our study was performed on endoscopic cytology brushes, which provides a less invasive method for biomarker assessment when compared with multiple biopsies. Brushes may better sample the entire Barrett’s segment too. In a previous study, we observed substantial interobserver agreement of FISH-based testing where complete agreement was achieved in 90% of the samples scored (κ for agreement of 0.77). [25]

As has been previously reported, the most informative clinical variables for predicting progression proved to be Barrett’s segment length and age. [19,26] Of the molecular markers, *p16* loss, *MYC* gain, and aneusomy were the strongest predictors of progression. We note that over-fitting is an important issue in biomarker progression studies in Barrett’s because of the inevitably small number of progressors in even large cohorts. We found that counting the number of abnormal markers among these three significant markers was an informative and consistent predictor of progression risk that reduced the risk of over-fitting our data due to

correlations between abnormalities in the individual markers (see online supplementary figure S7). When we added this 'Abnormal Marker Count' to a clinical model including age and Barrett's segment length this resulted in a model in which patients could be effectively classified into low and high-risk. The Abnormal Marker Count proved to be the strongest predictor in the multivariate analysis, while Barrett's length was the least significant predictor. Yet the circumferential Barrett's length did improve the sensitivity of the model. Possibly, molecular changes arising from small lesions in longer Barrett's segments may be missed because the abnormalities get 'diluted' and may not reach the variable threshold.

Using a risk score of '1', to minimise the number of false-negative patients, we could classify over half of the cohort as low-risk which had significantly better progression-free survival than patients in the high-risk group with a HR of 8.7 (95% CI 2.6 to 29.8). The sensitivity of our model was 86%, indicating that 86% of progressors would be correctly classified as high-risk. Nevertheless, the specificity was moderate at 54%, thus almost half of the non-progressors were misclassified as being high-risk. The positive predictive value was, given the overall low progression rate of non-dysplastic Barrett's patients, expected to be relatively low and was found to be 9%. Of importance was the high negative predictive value of 99% of the test indicating that 99% of the low-risk patients did not progress during the surveillance period (median 50 months).

The progression rate in our cohort was slightly higher than expected from previous reports in the literature with a combined progression rate of 0.97 for high-grade dysplasia/adenocarcinoma and 0.57 for adenocarcinoma alone. This may be partly explained by the fact that our study enrolled patients not only from non-academic hospitals but also from an academic tertiary referral centre for Barrett's oesophagus patients, which may have confounded the risk of progression. On the other hand, these rates correspond to outcomes of a recent meta-analysis that showed an estimated cancer incidence rate of 6.3/1000 person-years of follow-up.[4]

The predictive value of aneusomy is in accordance with the findings of two other large studies in which DNA content abnormalities, measured by image and flow cytometry, were found to be predictors of progression, although in mixed cohorts containing both low-grade dysplasia and nondysplastic cases.[10,12] *P16* loss has previously been reported as a predictor for progression but recent whole genome sequencing suggests that *p16* abnormalities are frequently found in Barrett's oesophagus but do not necessarily lead to malignancy.[8,12,27] However, the apparent discrepancy may be due to clonal expansion of *p16*-loss clones predisposing to subsequent evolution.[28] In our data, the average percentage of cells with *p16* loss was much higher in progressors when compared with non-progressors (10.6% vs 6.2%, $p=0.003$; Student's t-test) and *p16* contributed significantly to the risk of progression (table 2). It is noteworthy that, in contrast to our study, previous studies have reported loss or overexpression of *p53*, evaluated by LOH, mutation analysis and immunohistochemistry, as a significant predictor of progression.[12,29] However, most of these studies evaluated *p53* status in Barrett's oesophagus cohorts with various grades of dysplasia. In a recent case-control study, *p53* immunohistochemistry was not a significant predictor when analyses were restricted to patients with non-dysplastic Barrett's oesophagus.[10] In our analysis, *p53* loss was not a predictor and *p53* losses were rare events. The FISH

analysis as performed in this study may have underestimated *p53* losses, since *p53* locus loss may also occur in cells with genetic instability and gains for chromosome 17, which could lead to 'copy neutral loss of heterozygosity'. [30,31] Also, point mutations leading to abnormal *p53* function were undetectable by our methods. Other discrepancies might be due to differences in detection methods. [29,30]

We note that an evaluable brush could be obtained in few samples. A low cellular yield was the main limiting factor and in a few cases the analysis was hampered by the presence of clumps of cells that could not be scored due to overlapping cell nuclei. Clumps of cells can simply be prevented by vigorous shaking of the brush immediately following storage in the preservation medium. The quality of a sample can also be improved by the brushing technique; we note that the frequency of nonevaluable brushings decreased during the study period as endoscopists became more familiar with the brushing technique. We consider that experience will diminish this issue.

In a temporal case-cohort analysis, Li et al. showed that the interval between the occurrence of multiple genetic abnormalities and cancer development lies between 24 and 48 months. [16] We found a similar apparent 'window of opportunity' for biomarker prognostication in our cohort with a median progression interval of 34 months (IQR 24–38). It is interesting to extend the follow-up of our cohort to determine whether patients with a low-risk profile at baseline will remain progression-free over a longer period of time and maintain their low-risk status. Therefore, we would suggest that the most potential for clinical impact arising from this work is the ability to spare truly benign patients unnecessary surveillance. A hypothetical strategy may be that in low-risk patients the surveillance could be performed with increasing intervals for instance to 6 years, whereas high-risk patients, they could be advised to continue surveillance every 3 years (figure 4). Nevertheless, the implementation of such a strategy would require standardisation of processing and analysis of the biomarker assay and external validation of our findings in an independent cohort. DNA FISH is routinely applied in many standard pathology laboratories for both haematological malignancies and solid tumours. The assessment for *p16* loss, *MYC* gain and aneusomy can be performed in an efficient single multi-colour assay. DNA FISH is a labour-intensive technique and the implementation would imply substantial setup costs, but it has the potential for automation to increase laboratory efficiency. [15] Because the low-risk Barrett's population represents more than half of the participants, substantial economic benefits of applying this prediction model in surveillance programmes can be presumed. Although the study was not designed to perform a cost-efficacy analysis, we assume that an increased surveillance interval of 6 years for those that stratify as low-risk, which means skipping one endoscopy in >50% of the cohort, would eventually result in significant cost reduction.

In summary, our prospective cohort study suggests that a prediction model based on age, circumferential Barrett's segment length, and the molecular biomarkers *p16*, *MYC* and aneusomy determines progression risk in non-dysplastic Barrett's oesophagus. The use of this model identifies patients with a minimal risk of progression and therefore offers the potential to temper surveillance for low-risk Barrett's oesophagus patients without dysplasia. An external validation study is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AIC	Akaike's Index Criterion
AUC	area under the curve
CI	confidence interval
FISH	fluorescence in situ hybridisation
HR	hazard ratio
IQR	interquartile range

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Panel: Significance of this study**What is already known about this subject**

- The risk of oesophageal adenocarcinoma in patients with non-dysplastic Barrett's oesophagus is low and difficult to predict.
- Several genetic abnormalities have been studied to identify biomarkers that are associated with progression.
- However, high-quality, prospective studies evaluating the performance of biomarkers in nondysplastic Barrett's surveillance cohorts are lacking.

What are the new findings?

- In this large prospective cohort study, an Abnormal Marker Count combining abnormalities of *p16*, *MYC*, and aneusomy measured by fluorescence in situ hybridisation is an independent predictor of progression in patients with non-dysplastic Barrett's oesophagus.
- A prediction model including this Abnormal Marker Count is advantageous over a clinical model that uses only age and Barrett's length for long-term risk stratification.
- This is the largest prospective study to date, showing that long-term risk stratification for patients with non-dysplastic Barrett's oesophagus is possible using a prediction model based on the combination of clinical factors and genetic biomarkers.

How might it impact on clinical practice in the foreseeable future?

- This combined clinical and biomarker-based prediction model may be a useful tool to identify patients with a minimal risk of progression and therefore offers the potential to temper surveillance for low-risk Barrett's oesophagus patients.

Figure 1. Genetic abnormalities in non-dysplastic Barrett's oesophagus
(A) Violin plots of the percentage of cells showing abnormalities for the different markers in the baseline cohort (n=428). The width of the violin indicates the proportion of patients with the specified number of mutant cells, the black boxplots in the grey boxes indicate upper and lower quartiles, whiskers indicate fifthth and 95th percentiles. The white dots represent the median and the red squares represent the mean. The number below the graph indicates the number of patients that had at least one cell showing the marker. (B) The matrix shows the number of patients with abnormalities for each marker and possible pair of markers. The numbers in between ‘()’ are the numbers of progressors. Matrix cells are colour-coded from least frequent (yellow) to most frequent (dark green).

Figure 2. Distributions of area under the curve (AUC) and Akaike's Information Criterion (AIC) of the multivariate models when bootstrapping

The graph shows the AUC and AIC of (1) the *Clinical model* comprising all significant clinical variables on univariate analysis (age and Barrett's segment length), (2) the *All model* comprising clinical and all molecular markers, (3) the *Clinical+ molecular models*: a set of models with each individual marker that was significant on univariate analysis in combination with the clinical variables, (4) the *Clinical + Significant molecular markers* with all variables that were significant in the univariate analysis (ie, age, Barrett's segment length, p16, MYC, and aneusomy), and (5) the *Clinical + Abnormal Marker Count model* combining the significant clinical variables and the number of abnormal markers (p16 loss, MYC, and/or aneusomy) that a patient had at baseline. The model that was selected for further analysis (*Clinical + Abnormal Marker Count model*) is depicted with a star. AUC, Area Under the Curve; AIC, Akaike's Information Criterion; UVA, univariate analysis

Figure 3. Risk stratification of patients with non-dysplastic Barrett's oesophagus

(A) Receiver operating characteristic curves for the selected model '*Clinical + Abnormal Marker Count*' incorporating the variables age, circumferential Barrett's segment length and the Abnormal Marker Count and for the '*Clinical model*' consisting of the variables age and circumferential Barrett's segment length. Risk scores for the '*Clinical + Abnormal Marker Count*' model are indicated in red. (B) Kaplan-Meier curves for progression during follow-up in two groups (low-risk and high-risk) as defined by the '*Clinical + Abnormal Marker Count*' with a selected cut-off score of '1'.

NPV, negative predictive value.

Figure 4. A hypothetical workflow for the management of patients with non-dysplastic Barrett's oesophagus

(A) Conventional scheme of surveillance. (B) Scheme including the use of a prediction model based on the combination of clinical factors and genetic biomarkers.

FISH, fluorescence in situ hybridisation.

Table 1

Baseline characteristics of the study cohort

Characteristic	Study cohort
Subjects (n)	428
Age, year *	60 (51-67)
Male sex, n (%)	345 (81)
Body-mass index, kg/m ²	27.2 ± 3.9 [†]
Tobacco use, n (%)	294 (69)
Barrett's segment length *	
Circumferential Barrett's oesophagus, cm	2 (0-4)
Maximum Barrett's oesophagus, cm	3 (2-6)
Time since diagnosis of Barrett's oesophagus, year *	5 (2-10)
Use of proton-pump inhibitors, n (%)	422 (99)
Family history of Barrett's oesophagus, n (%)	52 (13)
Family history of oesophageal cancer, n (%)	39 (9)

Tobacco use (defined as ever smoking), use of proton-pump inhibitors and family history of Barrett's oesophagus and/or oesophageal cancer were self-reported.

* Data are shown as median (IQR).

[†] Mean ± SD

Table 2

Risk of progression among patients with non-dysplastic Barrett's oesophagus, according to clinical variables and genetic markers.

Variable	Variable type	Threshold for binary stratification	p Value	AIC	Median AUC	Hazard Ratio	95% CI
Age, year	Continuous		0.008	243.4	0.65	1.06	1.01 to 1.10
Male sex	Binary	M/F	0.29	249.9	0.22	1.01	1.00 to 1.01
BMI, kg/m ²	Continuous		0.68	248.7	0.54	0.98	0.88 to 1.09
Tobacco use	Binary	Yes / No	0.90	250.2	0.24	1.06	0.41 to 2.71
Family history of Barrett's oesophagus	Binary	Yes / No	0.55	225	0.15	1.4	0.46 to 4.21
Family history of oesophageal adenocarcinoma	Binary	Yes / No	0.13	223.5	0.16	2.32	0.77 to 6.99
Circumferential Barrett's length – cm	Continuous		0.01	245.9	0.55	1.15	1.03 to 1.29
p53 loss, %	Binary	0 / >0	0.18	249.2	0.28	1.01	1.00 to 1.01
p16 loss, %	Continuous		0.006	244.5	0.60	1.07	1.02 to 1.12
Her-2/neu gain, %	Continuous		0.89	250.9	0.07	1.02	0.73 to 1.44
20q gain, %	Binary	0 / >0	0.62	250.7	0.18	1	0.99 to 1.01
MYC gain, %	Binary	0 / >0	0.048	247.3	0.33	1.01	1.00 to 1.02
Aneusomy, %	Continuous		0.008	245.1	0.45	1.23	1.06 to 1.43
Abnormal Marker Count	Continuous		0.001	240.9	NA	1.91	1.29 to 2.81

*HR's and p values were calculated with the use of univariate Cox proportional-hazards analysis.

AIC, Akaike's Information Criterion; AUC, areaUnder the curve; HR, hazard ratio; BMI, body mass index; CI, confidence interval.

Table 3

Multivariate model predicting progression among patients with non-dysplastic Barrett's oesophagus.

Variable	Variable type	Mean	Hazard Ratio	Coefficient	95% Confidence Interval	p Value
Age	Continuous	58.82	1.06	0.054	1.01 to 1.10	0.01
Circumferential Barrett's length	Continuous	2.57	1.09	0.082	0.97 to 1.22	0.17
Abnormal Marker Count	Continuous	0.86	1.87	0.626	1.27 to 2.76	0.002

The Abnormal Marker Count was defined as the number of abnormal markers (*p16* loss, *MYC*, and/or aneusomy).

*HR's were calculated with the use of multivariate Cox proportional-hazards analysis.