Analysis of Dysplasia in Patients With Barrett's Esophagus Based on Expression Pattern of 90 Genes

Sibu Varghese,[1] Richard Newton,[2] Caryn S. Ross-Innes,[1] Pierre Lao-Sirieix,[1] Kausilia K. Krishnadath,[3] Maria O'Donovan,[1] M. Novelli,[4] Lorenz Wernisch,[2] Jacques Bergman,[3] and Rebecca C. Fitzgerald[1]

[1]*MRC Cancer Unit, University of Cambridge, Hutchison/MRC Research Centre, Cambridge, United Kingdom;* [2]*MRC Biostatistics Unit, Cambridge, United Kingdom;* [3]*Amsterdam Medical Center, Amsterdam, Netherlands; and* [4]*GI Services, University College Hospital, NHS Foundation Trust, London, United Kingdom*

BACKGROUND & AIMS: Diagnoses of dysplasia, based on histologic analyses, dictate management decisions for patients with Barrett's esophagus (BE). However, there is much intra- and inter-observer variation in identification of dysplasia—particularly low-grade dysplasia. We aimed to identify a biomarker that could be used to assign patients with low-grade dysplasia to a low- or high-risk group. **METHODS:** We performed a stringent histologic assessment of 150 frozen esophageal tissues samples collected from 4 centers in the United Kingdom (from 2000 through 2006). The following samples with homogeneous diagnoses were selected for gene expression profiling: 28 from patients with nondysplastic BE, 10 with low-grade dysplasia, 13 with high-grade dysplasia (HGD), and 8 from patients with esophageal adenocarcinoma. A leave-one-out cross-validation analysis was used identify a gene expression signature associated with HGD vs nondysplastic BE. Functional pathways associated with gene signature sets were identified using the MetaCore analysis. Gene expression signature sets were validated using gene expression data on BE and esophageal adenocarcinoma accessed through National Center for Biotechnology Information Gene Expression Omnibus, as well as a separate set of samples (n = 169) collected from patients who underwent endoscopy in the United Kingdom or the Netherlands and analyzed histologically. **RESULTS:** We identified an expression pattern of 90 genes that could separate nondysplastic BE tissues from those with HGD ($P < .0001$). Genes in a pathway regulated by retinoic acid–regulated nuclear protein made the largest contribution to this gene set ($P < .0001$); the transcription factor MYC regulated at least 30% of genes within the signature ($P < .0001$). In the National Center for Biotechnology Information Gene Expression Omnibus validation set, the signature separated nondysplastic BE samples from esophageal adenocarcinoma samples ($P = .0012$). In the UK validation cohort, the signature identified dysplastic tissues with an area under the curve value of 0.87 (95% confidence interval: 0.82–0.93). Of samples with low-grade dysplasia (LGD), 64% were considered high risk according to the 90-gene signature; these patients had a higher rate of disease progression than those with a signature categorized as low risk ($P = .047$). **CONCLUSIONS:** We identified an expression pattern of 90 genes in esophageal tissues of patients with BE that was associated with low- or high-risk for disease progression. This pattern might be used in combination with histologic analysis of biopsy samples to stratify patients for treatment. It would be most beneficial for analysis of patients without definitive evidence of HGD but for whom early endoscopic intervention is warranted.

Keywords: Biomarker; Esophageal Cancer; Diagnostic; Detection.

Barrett's esophagus (BE) has a highly variable outcome with 0.12%–0.5% of patients per year progressing to esophageal adenocarcinoma (EA).[1–4] The long-term survival of patients diagnosed with symptomatic EA remains poor. The purpose of endoscopic surveillance in patients with BE is to identify those at risk of progressing to cancer at an early, curable stage. Currently, this relies on the histopathologic diagnosis of dysplasia. The grading of dysplasia is based on the Vienna classification,[5] which takes into account a number of cytologic and tissue architectural features in the sample. The assessment of these features can be subjective and contribute to considerable intra- and inter-observer variability in the reporting of dysplasia. Low-grade dysplasia (LGD) has been shown to be overdiagnosed commonly by general pathologists, with high levels of variability between pathologists. Curvers et al[6] demonstrated that only 15% of BE cases diagnosed with LGD were confirmed to contain LGD when reviewed by 2 expert gastrointestinal pathologists, suggesting that 85% of patients were overdiagnosed. Importantly, the incidence of high-grade dysplasia (HGD) or cancer was 13.4% per patient per year for those in whom the diagnosis of LGD was confirmed, compared with 0.49% per patient per year for those who, after a consensus review, were downgraded to nondysplastic Barrett's esophagus (NDBE).[6] Another study reported a cancer incidence rate of 0.44% per year in those diagnosed with LGD. In this case, however, expert pathology review did not influence patient outcome, although the κ value among pathologists for the diagnosis of LGD in this study was worryingly low at 0.14, confirming the difficulty in assigning this diagnosis.[7] Several other studies spanning >20 years have highlighted the inter-observer

Abbreviations used in this paper: **AUC, area under the receiver operator curve; BE, Barrett's esophagus; CI, confidence interval; EA, esophageal adenocarcinoma; HGD, high-grade dysplasia; LGD, low-grade dysplasia; mRNA, messenger RNA; NDBE, nondysplastic Barrett's esophagus; NHS, National Health Service; SVM, support vector machine.**

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variability in the diagnosis of dysplasia in BE.[8–11] Given that LGD is currently the only accepted predictor for neoplastic progression before the point of intervention,[12] it is crucial to identify this group of true LGD patients in a more definitive manner. The interim results from a randomized controlled trial suggest that there is a significantly reduced risk of neoplastic progression in stringently confirmed LGD cases that were treated with radiofrequency ablation.[13] If this high-risk group can be identified with more certainty, then there would likely be a case for more widespread acceptance for treatment of patients at this early stage with ablative therapy.[14,15] As dysplasia is the cellular manifestation of multiple underlying genetic changes, a more direct measure of molecular factors might logically be a better indicator of cancer risk. Depending on the assay, a molecular test would also have the potential to provide more objective risk stratification than the current histologic assessment of dysplasia. In various pathologic contexts, the expression patterns of genes from microarray data have been shown to be powerful tools as biomarkers using the class prediction model. The class prediction model refers to formulating a rule with a set of genes often called a "gene signature" or "classifier" that can distinguish different classes of disease. A combination of levels or weights applied to the genes yields a score. If a score is above a certain threshold the specimen would be classified into one category, and if the score is below the threshold it would fall into the other category.

Such gene signatures have been shown to be useful in classifying different types of tumors,[16] predicting response to chemotherapy[17] and outcomes.[18–20] The breast cancer gene expression signature is an example whereby a microarray-based signature proved to be a more powerful predictor of disease outcomes than other clinical parameters.[20] Another example is in the characterization of thyroid nodules. A prospective multicenter study showed that a microarray based gene expression signature was a powerful tool in classifying thyroid nodules with indeterminate cytology on fine-needle aspiration.[21] Of the 265 indeterminate nodules, 85 were ultimately proven to be malignant, and the gene signature identified 78 of them correctly (92% sensitivity [95% confidence interval [CI]: 84−97); 52% specificity [95% CI: 44−59]). Management of thyroid nodules with indeterminate cytology poses a dilemma to the clinician. Use of this gene signature would appropriately favor the conservative approach in a majority of patients. This is analogous to the aim of our study in which we set out to identify a more objective biomarker for LGD, which is notoriously difficult to grade accurately, with the idea that this approach could be used as an adjunct to histopathology and thereby inform decision making with regard to the optimal surveillance intervals and the suitability of a patient for ablative therapy.

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Methods

Microarray Gene Expression Profiling (Training Set)

Fresh frozen esophageal samples (n = 150) were obtained between 2000 and 2006 from the following centers in the United Kingdom: Cambridge University Hospitals National Health Service (NHS) Foundation Trust, Cambridge; University College London Hospitals NHS Foundation Trust, London; Foundation Trust, Bristol; and Gloucestershire Hospitals NHS Foundation Trust, Gloucester. All samples were taken after endoscopy or surgery from consented patients at different stages of Barrett's neoplastic progression after approval by local ethical committees. A frozen section from each frozen sample used for molecular profiling was taken for consensus histopathologic reporting by 2 expert gastrointestinal pathologists blinded to the diagnosis of the corresponding clinical biopsies. Samples were graded for dysplasia and cancer using the Vienna histologic classification.[5] In their review, the pathologists also correlated the frozen section with the corresponding clinical formalin-fixed, paraffin-embedded H&E sample to aid the diagnosis. Samples with at least 50% of the epithelial cells displaying the diagnosis of interest were taken forward (Figure 1). Messenger RNA (mRNA) was extracted using the PicoPure RNA isolation kit (Applied Biosystems, Carlsbad, CA) according to manufacturer's instructions. mRNA that passed the quality control (A260/A280 ratio >1.8; A260/A230 ratio >1.6) was amplified using MessageAmp II kit (Life Technologies, Carlsbad, CA). After in vitro transcription, the antisense RNA was purified using the MinElute kit (Qiagen, Hilden, Germany). Five micrograms of each sample and control were labeled with cyanine dyes (Cy3 or Cy5) and hybridized to complementary gene-specific probes on a custom Agilent microarray (44K 60-mer oligo-microarray; Agilent Technologies, Santa Clara, CA). Each sample was hybridized twice using a dye reversal strategy. The images were then scanned and the fluorescence intensities for each probe recorded. After normalization using the Universal Human Reference RNA and correction of array intensity data, the ratios of transcript abundance (experimental to control) were obtained. A detrending template composed of 470 reporter probes was used to remove data bias.

Generation of Gene Signature

The categories of NDBE and HGD were used to identify a classifier for dysplasia because they are the most clearcut histopathologic diagnoses. As there was considerable variability in the reporting of LGD, and because EA is a late stage with variable differentiation status that might confound gene expression, both these groups were eliminated from the training set but were used later to validate the classifier. This resulted in 28 NDBE and 13 HGD samples being used as a training set. For each sample in

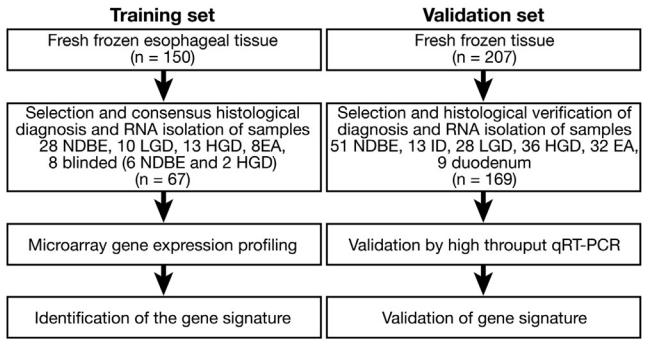

Figure 1. Study overview.