

The Molecular Signature of Normal Squamous Esophageal Epithelium Identifies the Presence of a Field Effect and Can Discriminate between Patients with Barrett's Esophagus and Patients with Barrett's-Associated Adenocarcinoma

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Abstract

Background and Aim: Genetic alterations in the normal tissues surrounding various cancers have been described, but a comprehensive analysis of this carcinogenic field effect in Barrett's-associated adenocarcinoma of the esophagus disease has not been reported. The aim of this study was to analyze the gene expression profile of a panel of highly selected genes in the normal squamous esophagus epithelium of patients with Barrett's esophagus, patients with Barrett's-associated adenocarcinoma, and a healthy control group to define the existence of a carcinogenic field effect, and to investigate the clinical importance of such a field effect in the management of Barrett's disease.

Methods: Forty-nine histologic normal squamous esophageal epithelia collected from 19 patients with Barrett's esophagus, 20 patients with Barrett's-associated esophageal adenocarcinoma, and a healthy control group of 10 patients were studied. A quantitative real-time reverse transcription-PCR method (TaqMan) was used to measure the expression

of a panel of genes with known associations with gastrointestinal carcinogenesis.

Results: A widespread carcinogenic field effect was detected for more than 50% of the genes analyzed including *Bax*, *BFT*, *CDX2*, *COX2*, *DAPK*, *DNMT1*, *GSTP1*, *RAR α* , *RAR γ* , *RXR α* , *RXR β* , *SPARC*, *TSPAN*, and *VEGF*. Based on the expression signature of the normal appearing squamous esophagus, a linear discriminant analysis was able to distinguish between the three groups of patients with an error rate of 0%.

Conclusion: This study provides the first comprehensive investigation of a carcinogenic field effect in Barrett's esophagus disease. Based on the gene expression signature of the normal esophagus, patients could be correctly characterized according to their pathologic classification by applying a linear discriminant analysis. Our results provide evidence that a molecular classification might have clinical importance for the diagnosis and treatment of patients with Barrett's esophagus disease. (Cancer Epidemiol Biomarkers Prev 2005;14(9):2113-7)

Introduction

The incidence of esophageal adenocarcinoma has increased significantly in the Western world and Europe over the last three decades (1). Esophageal adenocarcinoma is thought to evolve from a multistep process whereby the normal squamous epithelium is replaced by a specialized columnar epithelium in Barrett's esophagus, followed by progression through low-grade and high-grade dysplasia to adenocarcinoma (2). Although upper gastrointestinal endoscopy and ultrasound have improved preoperative staging, most esophageal adenocarcinomas present at an advanced stage, with an overall 5-year survival of only 10% to 20% (3). Refinements in existing

treatments are likely to produce only incremental improvements in survival. In contrast, significant survival benefits might result from developing methods to successfully detect cancer at an early stage, identifying those patients with Barrett's esophagus at greater risk of disease progression, or from the development of effective targeted therapies or chemoprevention strategies. The current endoscopic surveillance programs are based on a subjective histologic classification scheme with limited information about lesion biology and risk of progression to cancer.

In recent years, a variety of candidate biomarkers for discriminating between different stages of Barrett's esophagus and Barrett's-associated adenocarcinoma have been detected and show promise for monitoring or predicting progressive disease (4-14). Interestingly, some of the studies using normal squamous esophagus tissues from healthy patients as a negative control group suggest the existence of a genetic field effect in the presence of adenocarcinoma of the esophagus (8, 9, 11-13, 15, 16). The term "field cancerization" was first defined by Slaughter et al. in 1953 (17); it describes the existence of genetic alterations in histologically normal tissue surrounding malignant tumors. Since then, the so-called "field effect" has been detected for a variety of genetic and epigenetic events (15, 16, 18-21) and various cancers, including lung (20), colon (18), breast (21), and skin (19). However, a comprehensive analysis

Received 1/11/05; revised 6/21/05; accepted 7/15/05.

Grant support: Deutsche Krebshilfe/Dr. Mildred Scheel Stiftung (German Barrett Cancer Project, 70-2789-Si3, Teilprojekt G1b); P.M. Schneider, J. Brabender, R. Metzger, and S.E. Baldus) and Köln Fortune 63/2002 (J. Brabender).

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Note: J. Brabender and P. Marjoram contributed equally to this work.

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doi:10.1158/1055-9965.EPI-05-0014

of a carcinogenic field effect in Barrett's esophagus disease is lacking. We were recently able to show that a panel of highly selected genes can be used to distinguish between benign Barrett's esophagus and Barrett's-associated adenocarcinoma (14). We hypothesized that the expression profile based on the previous study can be used to differentiate between normal squamous esophageal mucosa patients with Barrett's esophagus, patients with Barrett's-associated adenocarcinoma, and a healthy control group, and eventually to define the existence and potential clinical importance of a carcinogenic field effect in this disease.

Materials and Methods

Tissue Samples for Reverse Transcription-PCR. Forty-nine normal squamous esophagus tissue samples obtained at endoscopy from 19 patients with Barrett's esophagus without adenocarcinoma, 20 patients with Barrett's-associated esophageal adenocarcinoma, and a healthy control group of patients without any disease of the esophagus were collected and immediately frozen in liquid nitrogen. There were 27 men and 22 women, with a median age of 58.1 years (range, 22-76 years). Endoscopic biopsies were done during routine endoscopy. Normal esophagus biopsies were taken at least 4 cm proximal to the macroscopically abnormal epithelium of patients with Barrett's esophagus or adenocarcinoma of the esophagus. Part of the specimen or an adjacent specimen was fixed in formalin and paraffin for histopathologic examination by a Barrett's expert pathologist. All specimens were classified histologically as normal squamous esophagus epithelium.

RNA Extraction and cDNA Synthesis. Total RNA was isolated by a single-step guanidinium isothiocyanate method using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) according to the instructions of the manufacturer, and cDNAs were prepared as described previously (7, 22). cDNA quantitation of the 23 genes of interest and the internal reference gene (*β-actin*) was done using a fluorescence detection method [ABI PRISM 7700 Sequence Detection System (TaqMan) Applied Biosystems, Foster City, CA] as described (23, 24). The PCR reaction mixture consisted of 600 nmol/L of each primer, 200 nmol/L probe, 5 units AmpliTaq Gold Polymerase, 200 μmol/L each dATP, dCTP, and dGTP, 400 μmol/L dUTP, 5.5 mmol/L MgCl₂, and 1× TaqMan Buffer A containing a reference dye, to a final volume of 25 μL (all reagents from Perkin-Elmer Applied Biosystems). Cycling conditions were 50°C for 10 seconds, 95°C for 10 minutes, followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute. The primers and probes used in this study for mRNA gene expression analysis (*Bax*, *BFT*, *COX2*, *DAPK*, *DNMT1*, *DNMT3a*, *DNMT3b*, *RARα*, *RARγ*, *RXRα*, *RXRβ*, *RXRγ*, *SPARC*, *TM3SF4*, *TP*, *GSTPI*, *CDX2*, *Bcl2*, *TSPAN*, *COX1*, *DPD*, and *ODC*) have been previously published (6, 14). Parallel TaqMan PCR reactions were done for each gene and the *β-actin* reference gene for each sample. All samples were analyzed with the full panel of genes and each reaction was done in triplicate. The ratio between the values obtained was used as a measure for the relative mRNA expression.

Statistical Analysis. TaqMan analyses yielded values that were expressed as ratios between two absolute measurements (gene of interest/internal reference gene). Gene expression levels in normal squamous esophagus tissues from the three groups of patients were compared using the Kruskal-Wallis test to identify significant differences in expressions levels. Linear discriminant analysis and logistic regression analysis were done to find genes or combinations of genes that have the power to discriminate between different histologies. Cross-validation studies and permutation tests were done to assess the success of the linear discriminant analysis (25).

Results

Gene Expression Analysis and Presence of Field Effect.

The median mRNA expressions of the panel of 23 genes in the three groups of patients are summarized in Table 1. As shown, there were substantial differences in mRNA expression in the different groups of patients. Accordingly, the genes can be grouped into six classes by their expression patterns. Class A genes are characterized by a low expression in the normal squamous esophageal epithelium of the control group, intermediate expression in the normal esophagus esophageal epithelium of the Barrett's group, and a maximum expression in the normal esophageal epithelium of the adenocarcinoma group. The expression levels in this subgroup are significantly different. Class B genes follow the same expression pattern as class A genes, but without statistically significant differences. Class C genes have the highest expression in the normal esophageal epithelium of the control group and are stepwise down-regulated in the Barrett's group and the adenocarcinoma group with statistical significance. Class D genes follow an "on-off" regulation. These genes have significantly lower expression levels in the normal esophageal mucosa of the control group and the adenocarcinoma group compared with the high expression in the normal esophageal epithelium of the Barrett's group. Class E genes follow the same pattern as class D genes, but with no significant differences between the three groups. Class F genes show no significant alterations in gene expression in the normal esophageal mucosa of the three groups. In total, 12 of 23 (52%) genes investigated show significantly different gene expression levels between the three groups of patients, suggesting the existence of a field effect.

Linear Discriminant Analysis and Permutation Test. To differentiate between our three groups of patients based on the gene expression signature in their corresponding normal squamous esophagus samples, we did a linear discriminant analysis on the full data set. This analysis aims to find linear combinations of gene expression levels, the so-called linear discriminant vectors, which can differentiate between the different samples (14, 25). The results (Table 2; Fig. 1) show that by using the linear discriminant vectors on the full panel

Table 1. Median and range mRNA expression in normal squamous esophagus tissues

Class	Gene	NE (CG group)	NE (BE group)	NE (EA group)	P
A	<i>Bax</i>	0.29 (0.73)	0.61 (0.91)	0.59 (1.15)	0.002*
	<i>BFT</i>	34.2 (41.9)	50.8 (40.5)	48.4 (186.9)	0.001*
	<i>COX2</i>	0.18 (1.56)	0.14 (0.66)	0.33 (1.53)	0.042*
	<i>DAPK</i>	0.003 (1.8)	0.005 (0.1)	0.11 (1.2)	0.02*
	<i>DNMT1</i>	0.11 (0.09)	0.34 (0.54)	0.31 (0.71)	0.001*
	<i>RARα</i>	0.15 (1.3)	0.13 (0.23)	0.22 (0.75)	0.04*
	<i>RXRα</i>	1.3 (1.2)	2.37 (8.3)	2.44 (9.0)	0.02*
	<i>RXRβ</i>	0.44 (0.92)	0.81 (0.97)	1.05 (2.13)	0.008*
	<i>SPARC</i>	0.58 (0.73)	1.07 (2.61)	1.79 (16.4)	0.039*
	B	<i>DNMT3a</i>	0.46 (1.3)	0.74 (0.88)	0.76 (1.67)
<i>RXRγ</i>		0.27 (0.69)	0.27 (2.23)	0.48 (4.48)	NS
<i>TM4SF3</i>		0.008 (0.01)	0.01 (1.28)	0.01 (0.18)	NS
<i>TP</i>		0.63 (2.1)	1.32 (5.1)	1.53 (7.6)	NS
C	<i>GSTPI</i>	4.5 (11.2)	4.13 (5.1)	2.9 (4.5)	0.03*
	<i>CDX2</i>	0.001 (0.004)	0.01 (0.004)	0.008 (0.72)	0.05*
D	<i>RARγ</i>	1.9 (4.4)	6.2 (5.9)	5.08 (7.2)	0.001*
	<i>Bcl2</i>	1.40 (6.2)	0.99 (2.43)	1.35 (5.6)	NS
E	<i>DNMT3b</i>	0.04 (0.18)	0.08 (0.20)	0.06 (0.15)	NS
	<i>TSPAN</i>	0.03 (0.72)	0.02 (0.48)	0.04 (0.61)	NS
	<i>VEGF</i>	0.63 (1.64)	1.32 (2.8)	1.02 (2.88)	NS
F	<i>COX1</i>	2.64 (5.2)	2.14 (3.8)	2.48 (4.36)	NS
	<i>DPD</i>	0.64 (1.2)	0.77 (2.0)	0.70 (2.2)	NS
	<i>ODC</i>	1.26 (5.15)	1.35 (8.46)	1.18 (2.52)	NS

Abbreviation: NE, normal esophagus; CG, control group; BE, Barrett's esophagus; EA, esophageal adenocarcinoma group. NS, not significant ($P > 0.05$). *Field effect detectable.

Table 2. Linear discriminant analysis for all samples and the full panel of genes

True histology	Predicted histology			Error rate
	NE (BE)	NE (EA)	NE (CG)	
NE (BE)	19	0	0	0
NE (EA)	0	20	0	0
NE (CG)	0	0	10	0
Overall				0

of genes, we are able to differentiate the three groups of patients based on the gene expression levels of the corresponding normal squamous esophageal epithelium with an error rate of 0.0. This result is striking because by simply classifying samples into groups at random, we would expect an error rate of 0.66. However, the issue of overfitting is very relevant here. Informally speaking, with so many genes, one might expect to classify anything with reasonable success, even if there was no direct relationship between gene expression and tissue type. Consequently, we assessed the success of the linear discriminant analysis by doing a cross-validation study as suggested by Dudoit et al. (25). In such a study, we leave out one of the samples and refit the linear discriminant analysis. We then use the new linear discriminant analysis fit to predict the type of the removed sample. This procedure is repeated for each possible sample that can be removed and we report the overall error rate for prediction of type for the removed samples. The results of the cross-validation study are shown in Table 3, which shows that the overall error rate was 0.16, which again is strikingly low. This verifies the strong signal present in our data. To further guard against overfitting, we did a permutation test. First, we created 100 data sets by randomly permuting the histologies on the actual data set. Thus, each of the 100 new data sets has a different, randomly permuted list of histologies, but the number of histologies of each type is constant across the data sets. We then did a linear discriminant analysis on the permuted data sets and compared the results to that which we got when doing an identical analysis on the original data. The results are illustrated in Fig. 2, where we see that the average classification error is 0.65 for the permuted data, compared with 0.16 when fitting the original data. These results fit with intuition. For randomly permuted data, there should be no relationship between groups of patients and gene

Table 3. Cross-validation study for all samples and the full panel of genes

True histology	Predicted histology			Error rate
	NE (BE)	NE (EA)	NE (CG)	
NE (BE)	14	3	2	0.26
NE (EA)	1	18	1	0.1
NE (CG)	1	0	9	0.1
Overall				0.16*

*The error rate is 0.66 if histologies were classified at random.

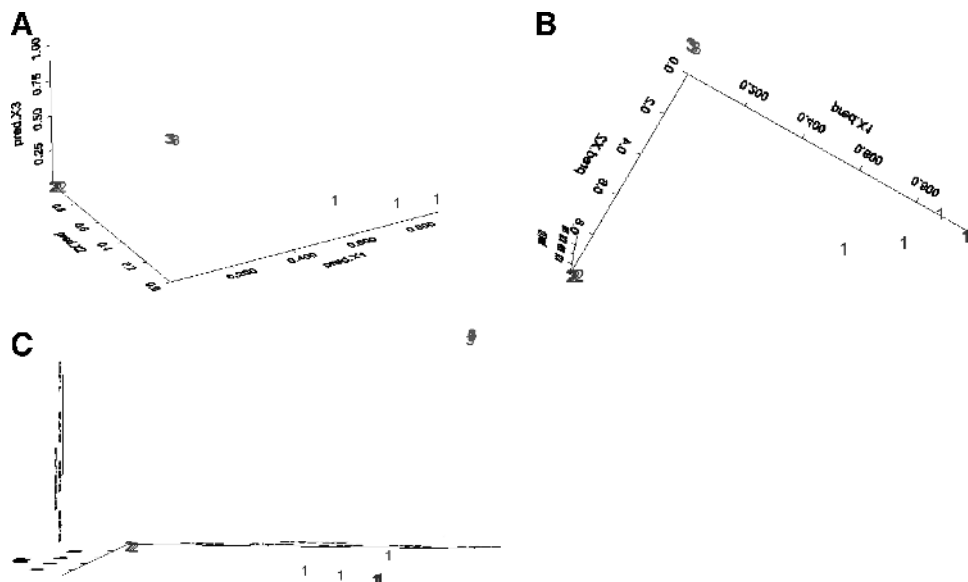
expression, and thus there should be no predictive power when attempting to assign a histology to the single, removed sample. Consequently, we regard the observed error rate of 0.16 in the cross-validation study as clear evidence of the existence of a strong signal in our data.

A linear discriminant analysis works optimally when data are approximately normally distributed, with constant mean and variance for each gene. However, as we have shown in previous work (14), a linear discriminant analysis is robust to departures from normality. We chose to normalize the data such that means and variances were constant across genes. However, we did not log-transform the data because the majority of the expression values are close to zero, thus making a log-transform problematic. Furthermore, gross departures from normality were not obvious. As a test of the robustness of our results to this decision, we have also conducted a linear discriminant analysis in which the data were logged and then normalized before analysis. The subsequent linear discriminant analysis worked comparably with the original linear discriminant analysis, with one less sample being correctly allocated in each of Tables 1 and 2 (results not shown).

Discussion

This study is the first to provide a comprehensive investigation of a carcinogenic field effect in the histologic normal squamous esophageal epithelium of patients with Barrett's esophagus and with Barrett's-associated adenocarcinoma. Most previous gene expression studies detecting a field effect in this disease have included only one or few genes. Although some of these studies identified the

Figure 1. Three two-dimensional representations of three-dimensional data from the linear discriminant analysis considering all samples and using the full panel of genes. Points are labeled according to their histologic group. The axes correspond to the first three linear discriminant vectors. Histologies: 1, normal squamous esophagus tissues of the Barrett's group; 2, normal squamous esophagus tissues of the esophageal adenocarcinoma group; 3, normal squamous esophagus tissues of the healthy control group.



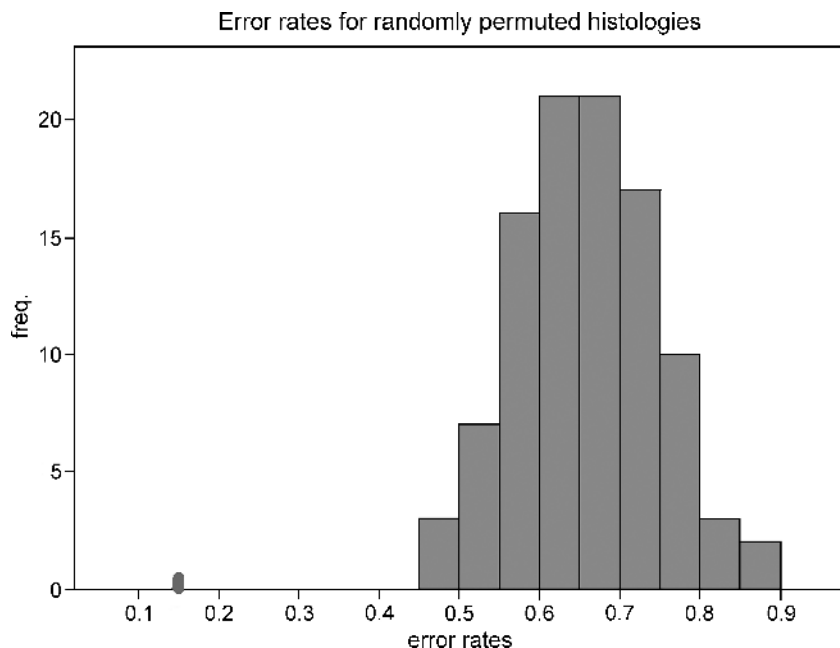


Figure 2. Histogram of the error rates for 100 randomly permuted data sets. The average classification error was 0.65 for the randomly permuted data compared with an error rate of 0.16 for the original data.

existence of a carcinogenic field effect in the normal esophagus tissues of patients with Barrett's adenocarcinoma, the potential clinical value of these findings was limited. In an attempt to overcome these limitations, we analyzed the mRNA expression of a panel of highly selected genes that have a known association with gastrointestinal carcinogenesis. Using this set of genes, our group was previously able to show that differences in gene expression levels can be used to distinguish between benign Barrett's esophagus and Barrett's-associated adenocarcinoma (14). The pattern of expression of many genes chosen was clearly different in the normal squamous esophagus tissues of our three groups of patients and resulted in the detection of a carcinogenic field effect in 52% of the genes analyzed. More than 3,300 single PCRs were done to analyze all samples with the full panel of genes in triplicate, resulting in a quantity of data which, although small in comparison with that generated by microarray studies, was nevertheless suitable for using bioinformatic methods, such as linear discriminant analysis.

By doing the linear discriminant analysis on our set of genes, we were able to completely distinguish between our three groups of patients (normal control group, Barrett's esophagus, and Barrett's cancer) based on the gene expression signature of their histopathologically normal-appearing squamous esophagus tissues. This result was not expected by us and is striking because it has potential clinical implications. Only a small proportion of Barrett's epithelium is usually taken as a biopsy specimen at endoscopy in routine clinical practice. Sampling error refers to the failure to detect pathology because of failure to biopsy the area containing the highest-grade lesion. As evidenced by the high frequency of cancer found in esophagectomy specimens from patients with a preoperative maximum diagnosis of Barrett's high-grade dysplasia, small areas of cancer are commonly not detected with endoscopic screening and conventional histopathologic methods (26, 27). If some histopathologically nonmalignant areas, for example the normal appearing squamous esophagus, have the expression signature according to our observed field effect, it may not be necessary to do a biopsy of the cancer itself to make the diagnosis of probable malignancy. We and others have recently shown that Barrett's esophagus can be discriminated from adenocarcinoma of the esophagus based on a specific gene expression profile (4, 14). Combination of these molecular approaches might be helpful in assisting the diagnostic process

and could thus theoretically reduce the risk of sampling error, which has to be shown in future trials.

It is common practice to use normal epithelial tissue adjacent to a carcinoma as a normal control when carcinoma-specific characteristics are investigated. This approach is frequent when immunohistochemical, mRNA, or protein expression studies are done. Tissue that is adjacent to tumor and that shows no macroscopic or microscopic abnormality is selected for comparison, as it is assumed to reflect normal tissue without cancer-related genotypic or phenotypic alterations. The results of this study and others indicate that tissue adjacent to carcinoma is not the appropriate control in a significant proportion of cases (8, 9, 11-13, 15, 16, 18-21, 28-30). It suggests that tissue surrounding tumors is not normal unless convincing evidence has been collected to show that it is. Adjacent epithelium must be checked for genetic abnormalities before it can be considered normal and a proper control for comparison with carcinoma.

The mechanism leading to the existence of the field effect in the normal esophageal mucosa of patients with adenocarcinoma and Barrett's esophagus is not known, but could follow a theory of action or reaction. In the case of action, due to an injurious environmental agent, for example the gastroesophageal refluxate, some of the early events of tumorigenesis in the normal esophagus have already occurred. In this case, these early events might predispose the apparently normal esophagus tissue to undergo further genetic changes leading ultimately to progression to adenocarcinoma. In the reaction theory, clones of abnormal cells, in the presence of cancer, have expanded widely throughout the mucosa to replace previously normal cells. In either case, it is apparent that genetic changes can precede the appearance of histopathologic changes in this disease.

In conclusion, our results suggest the existence of a widespread carcinogenic field effect in the normal squamous esophageal epithelium in the presence of Barrett's esophagus and Barrett's-associated adenocarcinoma. Using bioinformatic tools, patients can be correctly identified as healthy individuals, Barrett's esophagus patients, or cancer patients based on the gene expression signature of their normal squamous esophageal mucosa. Our results suggest that this molecular assay can be used to confirm a pathologic diagnosis or to help clarify an indeterminate diagnosis. Prospective clinical trials are warranted to determine the clinical value of this approach.

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