Published in final edited form as:

Gastroenterology. 2006 September ; 131(3): 925–933. doi:10.1053/j.gastro.2006.04.026.

Gene Expression Profiling Reveals Stromal Genes Expressed in Common Between Barrett's Esophagus and Adenocarcinoma

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Abstract

Background & Aims—Barrett's esophagus is a precursor of esophageal adenocarcinoma. DNA microarrays that enable a genome-wide assessment of gene expression enhance the identification of specific genes as well as gene expression patterns that are expressed by Barrett's esophagus and adenocarcinoma compared to normal tissues. Barrett's esophagus length has also been identified as a risk factor for progression to adenocarcinoma, but whether there are intrinsic biological differences between short and long-segment Barrett's esophagus can be explored with microarrays.

Methods—Gene expression profiles for endoscopically obtained biopsies of Barrett's esophagus or esophageal adenocarcinoma, and associated normal esophagus and duodenum were identified for 17 patients using DNA microarrays. Unsupervised and supervised approaches for data analysis defined similarities and differences between the tissues as well as correlations with clinical phenotypes.

Results—Each tissue displays a unique expression profile that distinguishes it from each other. Barrett's esophagus and esophageal adenocarcinoma express a unique set of stromal genes that is distinct from normal tissues, but similar to other cancers. Adenocarcinoma also showed lower and higher expression for many genes compared to Barrett's esophagus. No difference in gene expression was found between short and long-segment Barrett's esophagus.

Conclusions—The genome-wide assessment provided by current DNA microarrays reveals many candidate genes and patterns not previously identified. Stromal gene expression in Barrett's esophagus and adenocarcinoma are similar, indicating that these changes precede neoplasia.

INTRODUCTION

DNA microarrays provide the means to obtain a genome-wide assessment of gene expression. DNA microarrays have been previously used to compare esophageal adenocarcinoma, squamous cell carcinoma, and Barrett's esophagus, and established the presence of unique gene

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Grant Support: This study was supported by National Institutes of Health Award R01 DK063624 (A.W.L., G.T., and M.B.O.) and P30 DK56339 (Stanford Digestive Disease Center)

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expression profiles capable of discriminating between the tissues^{1–4}. In contrast to previous studies, which were limited by the diversity of genes and tissues examined, the present work provides a genome-wide determination of gene expression for Barrett's esophagus, esophageal adenocarcinoma, normal esophagus, and the duodenum. The duodenum serves as a control for the intestinal metaplasia characteristic of Barrett's esophagus.

Whether the higher cancer risk associated with long-segment Barrett's esophagus is secondary to intrinsic differences in biology or the extent of tissue involved is unknown⁵. Because differences in cell phenotype and physiology are associated with concomitant differences in gene expression, DNA microarrays are well-suited to evaluate whether differences exist between short and long segment Barrett's epithelium.

MATERIALS AND METHODS

Samples and RNA isolation

Unselected patients scheduled for endoscopic evaluation for Barrett's esophagus or esophageal adenocarcinoma were enrolled to participate in the study. Biopsies were obtained according to the Seattle protocol using a standard esophagogastroduodenoscope (Olympus GIF-XV10) and biopsy forceps (Radial Jaw 3, Boston Scientific Corp., Natick, MA). Four biopsies each were obtained from normal appearing esophagus (proximal to the Barrett's esophagus), salmon-colored Barrett's esophagus, adenocarcinoma (if present), and the duodenum. Twin biopsies were also obtained for each Barrett's esophagus sample and sent to pathology for analysis. Adenocarcinoma samples were also pathology confirmed. Duplicate microarrays were performed for one patient (sample 677 normal). All procedures were performed with patient consent and under approved human subjects protocols from Stanford University and the Palo Alto Veterans Affairs Health Care System.

Cell lines were derived from human esophageal adenocarcinomas associated with Barrett's metaplasia (Seg-1 and OE33), a poorly differentiated adenocarcinoma (TE7), and a squamous cell carcinoma (OE21). Seg-1⁶ cells (from Dr. David Beer, Univ. of Michigan) were grown at 5% CO₂ in DMEM with 4.5 g/L glucose, and L-glutamine (Cellgro, Mediatech, Inc., Herndon, VA), penicillin (100 U/ml), streptomycin (100 U/ml), and 10% fetal bovine serum. The OE21 and OE33⁷ cell lines (European Collection of Cell Cultures, Wiltshire, United Kingdom) and TE7⁸ cells (Dr. T. Nishihira, The Second Department of Surgery, Tohoku University School of Medicine, Japan) were grown in RPMI 1640 with 25mM Hepes, 10% fetal bovine serum, and penicillin and streptomycin (100 U/ml).

Total RNA (20–120µg) was obtained using Trizol (Invitrogen, Carlsbad, CA) and amplified one round as antisense RNA (Message AmpTM II, Ambion, Inc., Austin, TX). Commercial human reference RNA served as an internal standard (Universal Human Reference RNA, Stratagene Corp., La Jolla, CA).

Microarray procedure and data analysis

DNA microarrays were produced at the Stanford Functional Genomics Facility (www.microarray.org/) where protocols for the production of microarrays, array postprocessing, and hybridization can be found^{9, 10}. RNA from each sample was labeled with Cy5-dUTP and the RNA reference with Cy3-dUTP (Amersham Biosciences, GE Healthcare, Piscataway, NJ). Each microarray is composed of ~42,000 cDNA spots representing ~27,000 unique genes. Analysis of the scanned images was carried out using GenePix, version 5.0 (Axon Instruments, Molecular Devices Corp., Sunnyvale, CA) and spots of poor quality were flagged for exclusion from further analysis. The data was deposited in the Stanford Microarray

Database (<http://genome-www5.stanford.edu>) as the log base 2 ratio of the Cy5/Cy3 signal intensities and normalized with respect to the overall signal intensity for each channel¹¹.

Data analysis used an unsupervised hierarchical clustering algorithm that evaluates for similarities in gene expression patterns between individual genes and samples. The results were pseudocolored and visualized using Treeview¹². Cell line data was not included in the supervised or unsupervised analysis, but was projected alongside the tissue-derived data to assist in the identification of genes expressed by neoplastic cells. The cell line and tissue derived expression data did use the same common reference and were mean-centered together for each gene.

Statistically significant differences in gene expression between predesignated classes were assessed using a permutation of the t-statistic incorporated in the Significance Analysis of Microarrays software (SAM)¹³. Another supervised approach using shrunken centroid analysis, which is incorporated into the Prediction Analysis of Microarrays software (PAM), was used to identify genes capable of discriminating between predesignated classes¹⁴. Gene annotations were obtained using SOURCE (<http://source.stanford.edu>)¹⁵.

The entire dataset is available through the Stanford Microarray Database (genome-www5.stanford.edu) or the Lowe laboratory website (www.stanford.edu/group/lowelab) at Stanford University.

Real-time PCR and in situ hybridization

Real-time PCR was performed essentially as previously described¹⁶. Two-step real time PCR was used in which cDNA was first synthesized using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) followed by PCR amplification with iQTm SYBR Green Supermix (Bio-Rad Laboratories, Inc., Richmond, CA) and the iCycler iQTm Multicolor Real Time PCR Detection System (Bio-Rad). β -actin served as an internal control. Relative gene expression was determined using the Relative Expression Software Tool available at <http://www.gene-quantification.info/>¹⁷. *In situ* hybridizations were performed as previously described¹⁸.

RESULTS

Human subjects and cell lines

Gene expression was evaluated for endoscopically derived tissues obtained from Barrett's esophagus, esophageal adenocarcinoma, normal esophagus, and duodenum for 17 subjects (Table 1). One of 14 patients (sample 673) with Barrett's esophagus exhibited high-grade dysplasia. Samples from Barrett's esophagus adjacent to the tumor mass were obtained from two patients (samples B-1 and B-6).

Gene expression profiles were also determined for esophageal adenocarcinoma and squamous carcinoma cell lines. The gene expression profiles of cancer cell lines largely reflect the tumor of origin and thus were used to help define whether the source of gene expression was the neoplastic cell or associated mesenchymal cells¹⁹. The cell lines were not incorporated into unsupervised and supervised analysis because certain genes that are enhanced by *in vitro* propagation could potentially distort the clustering analysis.

Analysis of Gene Expression

An unsupervised hierarchical clustering approach was first used to characterize similarities in gene expression patterns between the 48 samples (Figure 1). Genes displaying similar expression patterns across many samples are clustered together in rows. Likewise, samples

were clustered in columns based on similarities in gene expression profiles. Except for 2 samples, the clustering was consistent with their tissue of origin as defined by endoscopy and pathology. Sample B-6 endoscopically appeared to be Barrett's but clustered with the adenocarcinomas and adjacent to the tumor derived from the same patient. Sample 680 clustered with Barrett's but was determined by pathology to be invasive poorly differentiated adenocarcinoma.

Gene clusters were identified that contained genes known to be specifically expressed by their tissue of origin (Figures 1 and 2). SAM analysis produced ranked lists of genes that are enriched in the normal esophagus, duodenum, Barrett's esophagus, and adenocarcinoma (Supplements 1 and 2). SAM analysis identified 648 genes with a 4-fold difference in expression between Barrett's and normal appearing esophagus. PAM analysis identified AGR2 alone as sufficient to accurately classify all the Barrett's esophagus samples from normal tissues (Supplement 2-sheets 2 and 3).

Prominent among the 157 genes significantly expressed at higher levels in Barrett's esophagus and adenocarcinomas are stromal genes such as the collagens (COL3A1, COL5A2, COL6A1, COL12A1) and CSPG2 (Supplement 2 – sheet 4). Recent studies identified a set of stromal genes whose expression possesses prognostic significance for patients with breast cancer²⁰. One hundred fifty-one of the genes were also expressed in the present dataset set and correctly classified 16 of 17 patient samples with Barrett's esophagus or adenocarcinoma within a single cluster (Figure 3). A cluster of 37 stromal genes showed higher expression in adenocarcinomas and Barrett's esophagus and not in normal esophageal tissue. *In situ* hybridization was performed for two stromal genes in this cluster, collagen 5A2 (COL5A2) and periostin (POSTN), that also ranked high for expression in adenocarcinomas and Barrett's esophagus by SAM analysis (Supplement 2). *In situ* hybridization showed enhanced expression in stromal cells for POSTN and COL5A2 (Figure 4).

SAM analysis identified 214 genes that are positively expressed at least 4-fold in adenocarcinomas compared to Barrett's esophagus (Supplement-2). The same analysis identified 829 genes that are negatively expressed in esophageal adenocarcinomas compared to Barrett's esophagus and includes genes implicated in other epithelial cancers as potential tumor suppressors (Table 2 and Supplement 2-sheet 5). Quantitative real time PCR confirmed the expression for 4 genes that may have a regulatory role and include up-regulated (DKK3 and BCAS1) and down-regulated genes (CHES1 and BRD2) in adenocarcinoma compared to Barrett's esophagus (Figure 5).

Comparison of short versus long-segment Barrett 's esophagus

Supervised and unsupervised analysis was performed between short and long-segment Barrett's esophagus samples. A total of 12 samples were studied with 6 long (average = 5.3 cm $\pm$ 2.5 S.D.) and 6 short (average = 1.6 cm $\pm$ 0.7 S.D.) segment Barrett's esophagus. Analysis with unsupervised hierarchical clustering and SAM analysis failed to identify any genes whose gene expression was significantly different between short and long Barrett's esophagus.

DISCUSSION

The expanded genome-wide coverage available with current microarrays was applied to normal, metaplastic, and neoplastic tissues. The resultant gene expression profiles were able to distinguish Barrett's esophagus, duodenum, normal esophagus, and esophageal adenocarcinoma from each other. For 2 patients, the samples were apparently misclassified. Barrett's esophagus is often heterogeneous, and because the pathology was inferred from an adjacent biopsy, variations based on sampling error are plausible.

Many genes overexpressed in Barrett's esophagus and adenocarcinoma, such as AGR2, BCAS1, and cyclooxygenase 2 (PTGS2), are also overexpressed by other epithelial cancers, suggesting mutual mechanisms in pathogenesis^{21–24}. These genes and others whose expression increases in esophageal adenocarcinomas compared to Barrett's esophagus serve as potential markers for risk stratification and diagnosis.

A striking finding is the stromal gene expression shared between adenocarcinoma and Barrett's esophagus, which is not observed in the normal esophagus or duodenum. The results indicate that stromal and extracellular matrix genes associated with tumor growth are expressed long before there is pathologic evidence of dysplasia. Although many stromal genes expressed in adenocarcinomas are likely to be expressed by associated mesenchymal cells and not neoplastic cells, they may still serve as excellent targets for diagnostic or therapeutic studies. Studies of other epithelial tumors support a significant role by the stroma in influencing tumor formation and growth^{25, 26}. Combined with the decreased expression observed for many genes, the results support a model in which the establishment of a specific extracellular matrix is an early event in carcinogenesis that precedes gene inactivation.

There were no differences in gene expression patterns between short and long-segment Barrett's esophagus. It is therefore unlikely that differences in cell type or cell physiology is responsible for the higher risk of transformation to adenocarcinoma by long-segment Barrett's esophagus. Alternative hypothesis include the overall extent of disease as a cause of increase risk rather than any intrinsic biological differences in the responsible cells.

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Figure 1.

Unsupervised hierarchical clustering analysis of normal and Barrett's esophagus, esophageal adenocarcinoma, and the duodenum. (A) Two-way hierarchical clustering of 48 tissue specimens and 13,090 genes. The image represents pseudocolored log₂ ratios of sample versus reference values for genes that have been filtered for spot quality, mean centered, and showing at least a 3-fold difference in expression over at least one array. Length of the dendritic tree represents the degree of similarity determined as previously described¹². The cell lines were not clustered and listed separately from the tissues. Clusters are labeled to the left of the data matrix and represent genes that are: "Normal" - enriched in the normal esophagus; "A" - enriched in Barrett's esophagus and adenocarcinoma; "B" - lower expression in

adenocarcinoma; “C” genes enriched in Barrett's esophagus and duodenum compared to the normal esophagus; “Duodenum” - enriched in the duodenum. Figure 1B - enlarged view of the array dendrogram shown in Figure 1A along with sample identification. Barrett's esophagus samples for which accurate lengths were available are labeled as long (L) or short (S) segment and used for subsequent analysis. *Top of page:* color bar depicting the $\log_2$ ratios and a color code for the samples. Grey boxes represent excluded data because of poor spot quality or insufficient signal. The primary dataset is available as in Supplement 4.

Figure 2.

Expanded view of the representative gene clusters shown in figure 1. Color code and experimental conditions are as described for figure 1. Two sections for cluster “B” are shown, one enriched in stromal genes (top) and the other that included cyclooxygenase 2 (PTGS2) (bottom).

Figure 3.

Unsupervised hierarchical clustering of samples and genes using 151 of 786 genes from reference (20) that met spot quality and showed at least a 4-fold difference in expression between arrays. At the top is a color bar depicting the $\log_2$ ratios and a color code for the samples. Grey boxes represent excluded data because of poor spot quality. The dendrogram is colored red for 37 genes clustered together that are highly expressed in Barrett's esophagus and esophageal adenocarcinoma.

Figure 4.

In situ hybridization for collagen 5A2 (COL5A2, panels a, b) and periostin (POSTN, panels c, d). RNA probes were generated with PCR for COL5A2 (forward 5'–GTATTGAGACACAAGGGGACCT–3' and reverse 5'–TTATTATTTTTCCTTTAATGATGGTG–3') and POSTN (forward 5'–TCCTGTTCCCAAGTCCAAA–3' and reverse 5'–TCAAATCGAAGAGTTGTGAACTG–3'). The resultant riboprobe was hybridized to sections of Barrett's esophagus (a, c) and normal appearing esophagus (b, d) from the same patient. Arrowheads point to collections of stromal cells. Magnification: 200X.

Figure 5.

Plot of expression results between cDNA microarray and real-time PCR of selected genes. Selected genes noted in the text that showed differential expression in esophageal adenocarcinoma compared to Barrett's esophagus were assayed with real-time PCR and plotted against the log₂ ratio of sample/reference derived from the microarray data. The genes selected were represented by only a single IMAGE clone on the microarrays. Samples assayed were N4, T4, N5, T5, N6, T6, 677N, 677B, 677D, 678N, 678B, and 678D. Primer sets used included 1) BRD2²⁷ (BRD2F - CAGGAACAGCTTCGGGCAGT and BRD2R - TCATGGGCCTGCTCTCTTCC); 2) DKK3²⁸ (DKK3F - ACATTGTTTCCATCTCCTCCCCTC and DKK3R - ACATTGTTTCCATCTCCTCCCCTC); 3) CHES1²⁹ (CHES1F - TGCCAATCACTCCCATTGGG and CHES1R - CCGCATCCGGCAGCTGG); 4) BCAS1³⁰ (BCAS1F - AGAAGGACTGGAGACTGCAAAG and BCAS1R - TAAGGTCAGCTGAAGTGGTGG); 5) ACTB (β-actin) (ACTBF - CGGGAAATCGTGCGTGACATTAAG and ACTBR - TGATCTCCTTCTGCATCCTGTCCG). Annealing temperatures for all primers was 60°C except for CHES1, which was 62°C.

Table 1

Number of subjects	17
average age	66.5 ± 11.7
males	16
Total samples	48
normal tissue	15
duodenum	14
Barrett's esophagus	14
with dysplasia	1
adenocarcinoma	5

Table 2

Genes associated with other cancers that are negatively expressed in esophageal adenocarcinoma

Gene Symbol	Gene Name	Ref
CHES1	checkpoint suppressor 1	31, 32
CDH1	E-cadherin	33–35
APC	adenomatosis polyposis coli	36, 37
BRD2	bromodomain-containing protein 2	38
BBX	bobby sox homolog	39
FOXP1	forkhead box P1	40
STK4	serine/threonine kinase 4	41, 42
HIPK1	homeodomain interacting protein kinase 1	43
FOXO3A	forkhead box O3A	44