Research Article

Cancer Epidemiology, Biomarkers & Prevention

# MicroRNA Profiles of Barrett's Esophagus and Esophageal Adenocarcinoma: Differences in Glandular Non-native Epithelium

Jennifer Drahos[1], Katrin Schwameis[2], Linda D. Orzolek[3], Haiping Hao[3], Peter Birner[2], Phillip R. Taylor[1], Ruth M. Pfeiffer[1], Sebastian F. Schoppmann[2], and Michael B. Cook[1]

## Abstract

**Background:** The tissue specificity and robustness of miRNAs may aid risk prediction in individuals diagnosed with Barrett's esophagus. As an initial step, we assessed whether miRNAs can positively distinguish esophageal adenocarcinoma from the precursor metaplasia Barrett's esophagus.

**Methods:** In a case–control study of 150 esophageal adenocarcinomas frequency matched to 148 Barrett's esophagus cases, we quantitated expression of 800 human miRNAs in formalin-fixed paraffin-embedded tissue RNA using NanoString miRNA v2. We tested differences in detection by case group using the $\chi^2$ test and differences in expression using the Wilcoxon rank-sum test. Bonferroni-corrected statistical significance threshold was set at $P < 6.25E{-}05$. Sensitivity and specificity were assessed for the most significant miRNAs using 5-fold cross-validation.

**Results:** We observed 46 distinct miRNAs significantly increased in esophageal adenocarcinoma compared with Barrett's esophagus, 35 of which remained when restricted to T1b and T2 malignancies. Three miRNAs (miR-663b, miR-421, and miR-502-5p) were detected in >80% esophageal adenocarcinoma, but <20% of Barrett's esophagus. Seven miRNAs (miR-4286, miR-630, miR-575, miR-494, miR-320e, miR-4488, and miR-4508) exhibited the most extreme differences in expression with >5-fold increases. Using 5-fold cross-validation, we repeated feature (miR) selection and case–control prediction and computed performance criteria. Each of the five folds selected the same top 10 miRNAs, which, together, provided 98% sensitivity and 95% specificity.

**Conclusion:** This study provides evidence that tissue miRNA profiles can discriminate esophageal adenocarcinoma from Barrett's esophagus. This large analysis has identified miRNAs that merit further investigation in relation to pathogenesis and diagnosis of esophageal adenocarcinoma.

**Impact:** These candidate miRNAs may provide a means for improved risk stratification and more cost-effective surveillance. *Cancer Epidemiol Biomarkers Prev; 25(3); 429–37. ©2015 AACR.*

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## Introduction

Esophageal adenocarcinoma is a highly lethal malignancy. A majority of patients present with late-stage disease, resulting in a 5-year survival rate of less than 20% (1). Incidence of this lethal malignancy has dramatically increased during the last three decades in the United States and continues to rise (2, 3). Prevention and control of esophageal adenocarcinoma could be enhanced with improved risk prediction and early diagnosis. The precursor metaplasia, which precedes esophageal adenocarcinoma, is known as Barrett's esophagus. Barrett's esophagus increases the risk of esophageal adenocarcinoma by 10- to 40-fold that of the general population, which translates to an absolute risk of approximately 0.5% per year or 1/200 person-years (4, 5). To make surveillance programs cost-effective, new strategies and biomarkers that can accurately distinguish these glandular epithelia are needed.

miRNAs represent a broad class of small RNA molecules, typically comprised of 18 to 22 nucleotides, which negatively regulate the translation and stability of target messenger RNAs. Each miRNA has the potential to regulate a diverse array of gene transcripts, and, as such, miRNAs have central roles in endogenous processes, including metabolism, inflammation, and carcinogenesis. Accumulating evidence indicates that miRNAs likely contribute to the pathogenesis of all human malignancies (6) with varied effects that include both tumor suppression and oncogenesis (7). Identifying miRNA signatures of cancer therefore could have utility for risk stratification and early detection. Although prior studies have identified several miRNAs associated with esophageal adenocarcinoma, no study to date has quantitated a wide range of miRNAs in >40 samples in an attempt to distinguish between the glandular epithelia of Barrett's esophagus and esophageal adenocarcinoma (8–15). Distinguishing such tissues using miRNA profiles may enhance the cost-effectiveness of surveillance programs. Therefore, our study aimed to discover miRNA signatures that could discriminate esophageal adenocarcinoma from Barrett's esophagus.

[1]Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DDHS, Bethesda, Maryland. [2]Department of Surgery, Upper-GI-Service, CCC-GET Unit, Medical University of Vienna, Vienna, Austria. [3]Johns Hopkins Medical Institutions Deep Sequencing and Microarray Core, Baltimore, Maryland.

**Note:** Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

J. Drahos and K. Schwameis are co-first authors of this article.

S.F. Schoppmann and M.B. Cook are co-last authors of this article.

**Corresponding Author:** Jennifer Drahos, National Cancer Institute, 9609 Medical Center Drive, Rm 7E-226, MSC 9774, Bethesda, MD 20892. Phone: 240-276-7298; E-mail: jennifer.drahos@nih.gov

**doi:** 10.1158/1055-9965.EPI-15-0161

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## Materials and Methods

### Study population

The case population consisted of 150 individuals with pathologically confirmed esophageal adenocarcinoma. Cases were randomly selected from all patients receiving surgical resection of adenocarcinoma of the esophagus at the Department of Surgery at Medical University of Vienna, Austria, who consented to participate in genetic research. Cases underwent surgical resection of the esophagus between 1992 and 2009, with macrodissection of the cancerous tissue prior to being processed as formalin-fixed paraffin-embedded (FFPE) samples. Cases were excluded from study selection if there was insufficient tumor tissue from surgical specimen for RNA isolation (explained in more detail below). No other specific inclusion or exclusion criteria were applied. The comparison population consisted of 150 randomly selected nondysplastic Barrett's esophagus patients at the same institution during 1992 to 2012 without esophageal adenocarcinoma using American College of Gastroenterology guidelines (16). Barrett's esophagus patients were frequency-matched on birth year (±5 years) and sex to the esophageal adenocarcinoma case population. All selected Barrett's esophagus patients had jumbo-forceps biopsies taken from a macroscopically visible (salmon-pink mucosa) metaplastic segment of the tubular esophagus, which was subsequently microscopically confirmed to have goblet cells. Two Barrett's esophagus patient samples failed to provide adequate RNA material, leaving 148 Barrett's esophagus and 150 esophageal adenocarcinoma samples for analysis.

### RNA isolation and quality control

For RNA extraction, FFPE blocks were cut in 10-μm scrolls at the Medical University of Vienna. The lesion of interest [esophageal adenocarcinoma or Barrett's esophagus (specialized intestinal metaplasia)] occupied ≥80% of the surface area of the blocks. Based on run-in experiments, a single 10-μm scroll was collected for esophageal adenocarcinoma cases and two 10-μm scrolls were collected for Barrett's esophagus biopsies. Samples were sent to the Johns Hopkins Medical Institute (JHMI) Deep Sequencing and Core Facility. The QIAGEN RNeasy FFPE Kit was used to extract total RNA (Qiagen).

### NanoString nCounter analysis

Total RNA samples were processed according to the manufacturer's protocol for the nCounter Human miRNA Expression Assay v2 Kit (NanoString). We used 175 ng of each total RNA sample as input into the nCounter Human miRNA sample preparation. Hybridization with the capture probe set was incubated for 16 hours. Data were extracted using the nCounter RCC Collector and were outputted as absolute counts. Detectable levels of nonspecific binding ("background noise") were measured by six negative controls for each sample, the mean plus two SDs were subtracted from each miRNA count for a given patient sample. Values at or below the background noise of a given sample were recoded to undetected for the qualitative analysis and missing for the quantitative analysis.

### Normalization of miRNAs

miRNA normalization factors were calculated based on a global mean normalization method first introduced to normalize data from RT-qPCR miRNA profiling studies in which a large number of miRNAs are tested per sample. This method has been shown to reduce overall variation better than endogenous invariant reference normalization and is advocated by NanoString: The mean count (expression) of each miRNA was calculated across all samples and then the miRNAs were ranked from highest to lowest mean count. The 100 miRNAs with the highest mean count were used to calculate the normalization factor. For each sample, the geometric mean of these 100 miRNAs was calculated. The arithmetic mean of all sample geometric means was also calculated (a constant). The normalization factor for each sample was determined by the ratio: arithmetic mean of sample geometric means/sample geometric mean. Each sample-specific normalization factor was applied to all miRNA counts for that sample. This dataset is referred to as the normalized dataset.

### Statistical analysis

To compare miRNA profiles between esophageal adenocarcinoma and Barrett's esophagus tissues, we first assessed differences in detection versus nondetection in the raw data by calculating the proportion of individuals that expressed the miRNA above background noise. The $\chi^2$ test was used to compare proportions of detect/nondetect in the two tissue groups. Secondly, the normalized dataset was used to calculate the median and interquartile range of each miRNA expressed in >30% of individuals in Barrett's esophagus and esophageal adenocarcinoma groups. The Wilcoxon rank-sum test was used to characterize the extent of differences in expression between groups. All tests were two-sided. Based on the 800 miRNA probes, the Bonferroni-corrected *P* value of <6.25E−5 was considered statistically significant.

We used 5-fold cross-validation to obtain unbiased estimates of performance criteria for a model that predicted esophageal adenocarcinoma or Barrett's esophagus status based on the most significant miRNAs (17). We randomly assigned subjects to five mutually exclusive groups with approximately equal numbers of cases and controls in each group. For a given fold, we used the four retained groups to select the top five miRNAs based on lowest *P* values of those <6.25E−5 and more frequent detection in esophageal adenocarcinoma versus Barrett's esophagus, and the top five miRNAs from the quantitative analysis based on greatest increased median fold expression in esophageal adenocarcinoma versus Barrett's esophagus with *P* values <6.25E−5. We entered these selected 10 miRNAs into a logistic regression model from which we estimated prediction probabilities for the group that was omitted from the fold. miRNAs selected from the detectability analysis were modeled as dichotomous variables (detected/not detected), and miRNAs selected from the quantitative analysis were modeled as log base 2 continuous. miRNAs below the background noise of a given sample were recoded to the mean of the six negative controls for that sample. We repeated this feature (miRNA) selection and prediction procedure five times, each time sequentially omitting a single distinct group of subjects to estimate unbiased prediction probabilities of case–control status. Prediction probabilities of ≥0.5 were interpreted to indicate case status. We used predicted case status to estimate sensitivity, specificity, and area under the receiver operating characteristic curve (AUC). All data analyses were performed using STATA version 13.0 (StataCorp LP).

### Predicted dysregulated mRNA targets and KEGG pathways

We used two Web-based bioinformatics tools, DIANA-microT web-server v5.0 (refs. 18, 19; based on Ensembl v69 and miRBase v18) and TargetScan 6.2 (20–23), to predict putative mRNA

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targets of the top ten miRNAs that positively identified esophageal adenocarcinoma cases from Barrett's esophagus in the detectability or quantitative analyses. We report potential mRNA targets from each of the two Web-based bioinformatic tools because they use different algorithms for prediction. The top mRNAs predicted from DIANA-microT web-server v5.0 were ordered and selected by the prediction score. Similarly, mRNAs predicted by TargetScan 6.2 were ordered and selected using the context score. Prediction and context scores are based on the tool-specific algorithms and reflect the confidence of the prediction.

To identify potential biologic pathways associated with the 10 most significant miRNAs (top five miRNAs from each analysis), we used DIANA miRPath v2.0, which can call on either of two databases to make predictions: microT-CDS (database of predicted mRNA targets) or TarBase v6.0 (database of experimentally verified mRNA targets). For miRNAs with less than five experimentally validated mRNA targets, we used the microT-CDS database. For miRNAs with five or more experimentally validated mRNA targets, we used the TarBase v6.0 databases. We report identified KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways with FDR-corrected $P$ values of <0.01 (24), except for miRNAs that targeted >10 KEGG pathways. For this subset of miRNAs, we used a more stringent $P$ value < 0.001 to limit the number of pathways reported.

## Results

Of the 150 esophageal adenocarcinoma cases, 79% were male, the median age of diagnosis was 65 years (interquartile range, 56–73), and the stage distribution was as follows: 9% ($n = 13$) stage 1B, 25% ($n = 36$) stage 2, 61% ($n = 86$) stage 3, and 5% ($n = 7$) stage 4 (Supplementary Table S1). Of the 148 frequency-matched Barrett's esophagus cases, 79% were also male and the median age at tissue collection was 69 (interquartile range, 62–78).

### Differences in miRNA detectability

Among 800 human miRNAs evaluated as part of the qualitative analysis, there were 27 miRNAs detected in a significantly greater proportion of esophageal adenocarcinoma cases compared with Barrett's esophagus cases (Table 1). Of these miRNAs, six are part of the high confidence subset of miRBase entries (25). The largest difference in detection was observed for miR-421, which was detected in 98% of cancer tissues compared with just 16% of Barrett's esophagus tissues. Strikingly different detection rates were also observed for miR-663b (80% vs. 3%), miR-502-5p (84% vs. 11%), miR-1915-3p (97% vs. 32%), miR-601 (98% vs. 41%), and miR-187-3p (81% vs. 27%). Some miRNAs were not detected in any Barrett's esophagus cases (miR-206, miR-600, miR-1305, and miR-371a-5p) but had low-to-moderate expression in esophageal adenocarcinoma cases (13%–33% detectable).

In Barrett's esophagus cases, we observed 127 miRNAs with significantly greater detection rates compared with esophageal adenocarcinoma cases (Table 1 and Supplementary Table S2), the largest differences in detection of which were for miR-215 (59%), miR-574-3p (53%), and miR-31-5p (53%).

### Quantitative differences of miRNA expression

There were 24 miRNAs with a median expression $\geq$2-fold in esophageal adenocarcinoma tissues compared with Barrett's esophagus tissues, the largest of which was a 34-fold increased median expression of miR-4286 (Table 2 and Supplementary Table S3). Six of the 24 miRNAs (miR-630, -575, -494, -320e, -4488, and -4508) exhibited >5-fold median differences. In combination, Tables 1 and 2 provide 46 unique miRNAs that were higher in esophageal adenocarcinoma than Barrett's esophagus cases.

There were 17 miRNAs that had a significantly greater median expression in Barrett's esophagus tissues compared with esophageal adenocarcinoma tissues (Table 2 and Supplementary Table S4). A majority of these median fold changes were in the range of 2 to 3. The largest increase, a 12-fold median expression in Barrett's esophagus tissues compared with esophageal adenocarcinoma, was observed for miR-205-5p.

Results from both the detectability and quantitative analyses were unaffected when we excluded the 36% of esophageal adenocarcinoma cases that received neoadjuvant chemotherapy or chemoradiation (results not shown). Results were robust when the esophageal adenocarcinoma case group was restricted to T1b and T2 malignancies, with 35 of the 46 miRNAs remaining statistically significantly increased in esophageal adenocarcinoma when compared with Barrett's esophagus (Supplementary Tables S5 and S6).

### Discriminatory ability of a miRNA signature

From each of the 5-fold cross-validation models, the same 10 miRNAs were repeatedly selected based on *a priori*–specified criteria. These were miR-663b, miR-421, miR-502-5p, miR-1915-3p, and miR-601 from the detectability analyses and miR-4286, miR-630, miR-575, miR-494, and miR-320e from the quantitative analyses. A model including these 10 miRNAs had 98% sensitivity, 95% specificity, and 0.97 AUC. We also assessed predictive performance of 45 models that included each pairwise combination of markers, which had AUC values ranging from 0.78 to 0.98 with lower confidence estimates of 0.73 to 0.96 (Supplementary Table S7).

### Predicted mRNA targets and biologic pathways

We selected the five most significant miRNAs from each main analysis for *in silico* assessment of mRNA targets using DIANA-microT-CDS web-server 5.0 and TargetScan 6.2. We used the DIANA-microT-CDS prediction score to order the 2,778 predicted mRNA targets of these 10 miRNAs, and then selected just the top 10 of each for presentation (Table 3). Similarly, we ordered by context score the 2,920 mRNA transcripts with conserved sites predicted by TargetScan and selected the top 10 mRNA targets for presentation (Table 3). Five of these miRNAs—630, 494, 663b, 421, and 502-5p—had one or more mRNA targets that have been validated by functional analyses of human tissues (Table 3). Biologic (KEGG) pathways predicted by DIANA miRPath v2.0 using the top 10 selected miRNAs that positively distinguished esophageal adenocarcinoma from Barrett's esophagus are shown in Table 3. Specific cancers were identified with mRNAs targeted by 10 to 15 miRNAs each, including prostate, colorectal, pancreatic, thyroid, bladder, glioma, and endometrial cancers. PI2K-AKT, mTOR, and mRNA surveillance signaling pathways were also enriched for targeted mRNAs by 9 to 18 distinct miRNAs.

## Discussion

We have elucidated a putative miRNA signature that appears to discriminate esophageal adenocarcinoma from Barrett's

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**Table 1.** miRNAs differentially detected among esophageal adenocarcinoma and Barrett's esophagus[a]

| | | Samples with detectable expression[b] | | | Median expression (p25, p75)[c] | |
|---|---|---|---|---|---|---|
| | | EA (%) | BE (%) | *P* value[d] | EA | BE |
| | **miRNA: EA > BE** | | | | | |
| | miR-663b | 80.0 | 2.7 | 1.8E−48 | 141 (74, 256) | 112 (60, 139) |
| | miR-421 | 98.0 | 15.5 | 7.1E−47 | 248 (114, 1079) | 44 (36, 63) |
| | miR-502-5p | 84.0 | 10.8 | 1.1E−36 | 146 (95, 257) | 96 (64, 100) |
| | miR-1915-3p | 97.3 | 31.8 | 2.2E−32 | 221 (107, 403) | 159 (118, 206) |
| | miR-601 | 98.0 | 41.2 | 1.3E−26 | 360 (172, 959) | 172 (55, 275) |
| | miR-187-3p | 81.3 | 27.0 | 4.9E−21 | 111 (61, 205) | 120 (101, 140) |
| | miR-206 | 33.3 | 0.0 | 1.3E−17 | 271 (171, 558) | 0 (0, 0) |
| | miR-320a | 40.7 | 2.7 | 4.4E−17 | 71 (42, 124) | 34 (29, 37) |
| | miR-1224-5p | 36.0 | 2.0 | 2.3E−15 | 171 (82, 254) | 72 (63, 111) |
| | miR-500b | 42.7 | 4.1 | 3.8E−15 | 126 (51, 196) | 42 (27, 59) |
| # | miR-193a-5p | 60.0 | 18.9 | 4.2E−13 | 82 (50, 136) | 40 (35, 46) |
| | miR-127-3p | 84.0 | 45.9 | 5.5E−12 | 124 (76, 212) | 54 (46, 67) |
| | miR-1290 | 76.7 | 37.2 | 5.7E−12 | 161 (100, 281) | 132 (69, 184) |
| | miR-944 | 26.7 | 1.4 | 2.7E−11 | 172 (95, 265) | 59 (43, 76) |
| | miR-1469 | 69.3 | 32.4 | 1.9E−10 | 140 (71, 209) | 280 (230, 351) |
| | miR-4532 | 87.3 | 55.4 | 1.0E−09 | 137 (79, 217) | 65 (44, 152) |
| | miR-3195 | 21.3 | 0.7 | 1.4E−09 | 99 (47, 136) | 52 (52, 52) |
| # | miR-769-5p | 62.7 | 28.4 | 2.8E−09 | 79 (44, 150) | 45 (35, 53) |
| | miR-600 | 15.3 | 0.0 | 9.6E−08 | 49 (38, 68) | 0 (0, 0) |
| # | miR-186-5p | 96.7 | 76.4 | 2.7E−07 | 203 (112, 358) | 89 (76, 103) |
| | miR-1305 | 13.3 | 0.0 | 9.7E−07 | 140 (112, 157) | 0 (0, 0) |
| | miR-663a | 84.7 | 59.5 | 1.2E−06 | 123 (67, 211) | 77 (46, 168) |
| | miR-4461 | 54.7 | 27.0 | 1.2E−06 | 80 (47, 156) | 71 (40, 96) |
| | miR-371a-5p | 12.7 | 0.0 | 2.1E−06 | 71 (42, 128) | 0 (0, 0) |
| # | miR-125a-3p | 55.3 | 28.4 | 2.4E−06 | 86 (50, 134) | 55 (43, 100) |
| | miR-4421 | 22.0 | 4.7 | 1.2E−05 | 43 (34, 52) | 41 (35, 82) |
| | miR-4508 | 98.7 | 85.1 | 1.8E−05 | 382 (207, 870) | 74 (50, 437) |
| | **miRNA: BE > EA**[a] | | | | | |
| | miR-215 | 30.7 | 89.9 | 1.8E−25 | 76 (58, 144) | 265 (150, 705) |
| # | miR-31-5p | 42.7 | 95.3 | 1.1E−22 | 69 (51, 107) | 138 (108, 175) |
| # | miR-98 | 47.3 | 98.0 | 1.3E−22 | 156 (117, 198) | 221 (185, 246) |
| | miR-107 | 45.3 | 95.9 | 1.0E−21 | 86 (68, 112) | 135 (112, 162) |
| # | let-7f-5p | 52.0 | 99.3 | 2.1E−21 | 434 (255, 607) | 666 (508, 826) |
| # | miR-32-5p | 47.3 | 95.9 | 1.5E−20 | 102 (76, 132) | 176 (130, 216) |
| # | miR-574-3p | 31.3% | 84.5% | 1.7E−20 | 50 (41, 59) | 91 (80, 108) |
| | miR-1178 | 46.0% | 94.6% | 5.0E−20 | 72 (60, 85) | 94 (75, 116) |
| | miR-3147 | 48.0% | 95.3% | 1.6E−19 | 69 (60, 84) | 92 (76, 113) |
| # | miR-582-5p | 18.0% | 68.2% | 1.9E−18 | 45 (35, 55) | 71 (59, 83) |
| # | miR-15b-5p | 59.3% | 100.0% | 3.3E−18 | 390 (251, 628) | 1161 (859, 1397) |
| | miR-24-3p | 55.3% | 98.0% | 3.9E−18 | 253 (183, 353) | 372 (279, 490) |
| | miR-28-5p | 53.3% | 96.6% | 7.3E−18 | 159 (127, 218) | 201 (161, 241) |
| | miR-18a-5p | 44.0% | 89.9% | 4.3E−17 | 62 (48, 91) | 84 (65, 107) |
| | miR-223-3p | 59.3% | 98.6% | 9.5E−17 | 343 (239, 587) | 343 (220, 552) |
| | miR-19b-3p | 59.3% | 98.6% | 9.5E−17 | 214 (162, 274) | 387 (295, 470) |
| # | miR-30b-5p | 62.7% | 100.0% | 1.6E−16 | 441 (305, 666) | 844 (603, 1202) |
| | miR-135b-5p | 58.7% | 98.0% | 2.1E−16 | 247 (150, 384) | 265 (176, 386) |
| # | miR-148a-3p | 64.0% | 100.0% | 7.2E−16 | 511 (336, 936) | 1855 (1295, 2560) |
| | miR-604 | 38.7% | 83.8% | 1.4E−15 | 50 (44, 62) | 64 (54, 90) |
| | miR-378g | 28.0% | 73.6% | 3.3E−15 | 51 (40, 80) | 85 (64, 115) |
| | miR-148b-3p | 64.0% | 99.3% | 3.9E−15 | 184 (155, 219) | 285 (235, 326) |
| | miR-95 | 38.7% | 83.1% | 4.0E−15 | 71 (61, 93) | 97 (74, 123) |
| | miR-26b-5p | 66.0% | 100.0% | 6.6E−15 | 661 (407, 883) | 1093 (817, 1397) |
| # | miR-150-5p | 66.7% | 100.0% | 1.4E−14 | 438 (252, 693) | 665 (419, 1075) |

Abbreviations: BE, Barrett's esophagus; EA, esophageal adenocarcinoma.

[a]Displaying top 25 of 127.

[b]miRNA detected above mean + 2 SDs of negative controls for individual samples.

[c]Normalized data with miRNA expression at or below background set to missing.

[d]Ordered by *P* value.

#Listed in high confidence miRNA dataset[25].

esophagus. The results from this discovery study clearly indicate stark differences in the miRNA expression profiles of these glandular tissue types. Using conservative thresholds, we found that 46 distinct miRNAs increased in esophageal adenocarcinoma compared with Barrett's esophagus, 35 of which were also statistically significantly increased when the esophageal adenocarcinoma case group was restricted to T1b and T2 malignancies. Notably, seven miRNAs (miR-4286, miR-630, miR-575, miR-494, miR-320e, miR-4488, and miR-4508) positively discriminated esophageal adenocarcinoma with fold increases >5. Our

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**Table 2.** miRNAs differentially expressed among esophageal adenocarcinoma and Barrett's esophagus

| | | Samples with detectable expression[a] | | Median expression (p25, p75)[b] | | | |
|---|---|---|---|---|---|---|---|
| | | EA (%) | BE (%) | EA | BE | *P* value | Median fold change[c] |
| | **miRNA: EA > BE** | | | | | | |
| | miR-4286 | 98.7 | 100.0 | 7185 (3192, 12792) | 211 (161, 436) | <2.0E−16 | 34.0 |
| | miR-630 | 100.0 | 93.2 | 2089 (744, 6031) | 111 (62, 943) | <2.0E−16 | 18.9 |
| | miR-575 | 100.0 | 93.2 | 1009 (424, 2400) | 97 (64, 595) | <2.0E−16 | 10.4 |
| | miR-494 | 100.0 | 100.0 | 11868 (7186, 22903) | 1726 (1071, 13719) | 5.8E−15 | 6.9 |
| | miR-320e | 100.0 | 99.3 | 691 (310, 1544) | 117 (87, 218) | <2.0E−16 | 5.9 |
| | miR-4488 | 99.3 | 95.3 | 487 (259, 912) | 88 (63, 424) | <2.0E−16 | 5.5 |
| | miR-4508 | 98.7 | 85.1 | 382 (207, 870) | 74 (50, 437) | 5.3E−14 | 5.2 |
| | miR-4516 | 100.0 | 100.0 | 819 (445, 1633) | 167 (110, 1131) | 2.4E−15 | 4.9 |
| | miR-1246 | 80.0 | 69.6 | 1026 (531, 2187) | 284 (119, 1632) | 7.0E−06 | 3.6 |
| | miR-125b-5p | 92.0 | 99.3 | 4031 (1616, 6673) | 1294 (938, 1900) | 1.8E−15 | 3.1 |
| | miR-151a-3p | 99.3 | 95.3 | 256 (129, 471) | 95 (82, 109) | <2.0E−16 | 2.7 |
| | miR-4284 | 75.3 | 63.5 | 177 (116, 243) | 71 (45, 165) | 1.7E−11 | 2.5 |
| | miR-100-5p | 97.3 | 97.3 | 586 (338, 987) | 244 (179, 327) | <2.0E−16 | 2.4 |
| | miR-143-3p | 76.0 | 97.3 | 4589 (2015, 10812) | 1928 (1253, 3099) | 2.1E−11 | 2.4 |
| | miR-127-3p | 84.0 | 45.9 | 124 (76, 212) | 54 (46, 67) | <2.0E−16 | 2.3 |
| # | miR-186-5p | 96.7 | 76.4 | 203 (112, 358) | 89 (76, 103) | <2.0E−16 | 2.3 |
| # | miR-181a-5p | 98.0 | 100.0 | 832 (511, 1366) | 370 (289, 483) | <2.0E−16 | 2.2 |
| | miR-1 | 42.7 | 48.6 | 132 (81, 210) | 62 (54, 81) | 1.8E−11 | 2.1 |
| | miR-4532 | 87.3 | 55.4 | 137 (79, 217) | 65 (44, 152) | 1.8E−05 | 2.1 |
| # | miR-218-5p | 47.3 | 43.9 | 127 (89, 183) | 61 (51, 84) | 7.1E−14 | 2.1 |
| | miR-601 | 98.0 | 41.2 | 360 (172, 959) | 172 (55, 275) | 7.0E−08 | 2.1 |
| # | miR-28-3p | 90.0 | 78.4 | 171 (122, 272) | 83 (71, 101) | <2.0E−16 | 2.1 |
| # | miR-574-5p | 98.7 | 99.3 | 221 (140, 330) | 108 (92, 141) | <2.0E−16 | 2.0 |
| # | miR-30a-5p | 99.3 | 98.6 | 811 (448, 1406) | 402 (294, 536) | <2.0E−16 | 2.0 |
| | **miRNA: BE > EA** | | | | | | |
| | miR-205-5p | 43.3 | 62.2 | 377 (180, 785) | 4579 (1459, 6885) | 3.1E−15 | 12.1 |
| | miR-194-5p | 70.0 | 100.0 | 774 (295, 1414) | 3319 (2044, 5426) | <2.0E−16 | 4.3 |
| | miR-203 | 67.3 | 99.3 | 300 (168, 615) | 1110 (407, 5439) | 3.3E−12 | 3.7 |
| # | miR-148a-3p | 64.0 | 100.0 | 511 (336, 936) | 1855 (1295, 2560) | <2.0E−16 | 3.6 |
| | miR-215 | 30.7 | 89.9 | 76 (58, 144) | 265 (150, 705) | 2.8E−11 | 3.5 |
| # | miR-15b-5p | 59.3 | 100.0 | 390 (251, 628) | 1161 (859, 1397) | <2.0E−16 | 3.0 |
| # | miR-192-5p | 82.0 | 100.0 | 388 (211, 679) | 1126 (694, 1755) | <2.0E−16 | 2.9 |
| | miR-720 | 96.0 | 100.0 | 6169 (2081, 9627) | 15500 (11554, 19369) | <2.0E−16 | 2.5 |
| | miR-1260a | 72.0 | 100.0 | 667 (375, 893) | 1638 (1239, 2140) | <2.0E−16 | 2.5 |
| | miR-200c-3p | 92.0 | 100.0 | 1430 (737, 2318) | 3272 (2744, 3708) | <2.0E−16 | 2.3 |
| | let7g-5p | 69.3 | 100.0 | 2371 (623, 3511) | 5346 (4358, 6232) | <2.0E−16 | 2.3 |
| | let7a-5p | 72.7 | 100.0 | 5475 (904, 7967) | 12094 (10248, 14002) | <2.0E−16 | 2.2 |
| | miR-375 | 85.3 | 100.0 | 154 (105, 239) | 328 (249, 460) | <2.0E−16 | 2.1 |
| | miR-200b-3p | 84.7 | 100.0 | 1034 (569, 1686) | 2195 (1794, 2759) | <2.0E−16 | 2.1 |
| # | miR-31-5p | 42.7 | 95.3 | 69 (51, 107) | 138 (108, 175) | 3.2E−12 | 2.0 |
| | miR-1469 | 69.3 | 32.4 | 140 (71, 209) | 280 (230, 351) | 2.1E−08 | 2.0 |
| # | miR-200a-3p | 78.7 | 100.0 | 591 (356, 1029) | 1178 (928, 1497) | 4.4E−16 | 2.0 |

Abbreviations: BE, Barrett's esophagus; EA, esophageal adenocarcinoma.

[a]miRNA detected above mean + 2 SDs of negative controls for individual samples.

[b]Normalized data with miRNA expression at or below background set to missing.

[c]Ordered by median fold change.

#Listed in high confidence miRNA dataset[25].

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results provide a broad catalog of altered miRNAs that merit further investigation in relation to the pathogenesis and diagnosis of esophageal adenocarcinoma.

Prior studies of miRNA signatures in Barrett's esophagus and esophageal adenocarcinoma tissues have not provided consistent results (refs. 8–15, 26–31; reviewed in refs. 32–35). Although small sample sizes and pathologic heterogeneity may have contributed to the inconsistency (36, 37), the choice of the comparison group is the likely driver of the disparate results (34). The aim of our study was to distinguish esophageal adenocarcinoma from Barrett's esophagus, as the miRNA profiles of these tissue types are likely to be fairly similar, given their mutual glandular phenotype, shared environment (distal esophagus), and the etiopathological associations. Distinguishing these similar phenotypes is essential for preventive efforts.

To date, eight previous studies also directly compared miRNAs of Barrett's esophagus and esophageal adenocarcinoma (8–15, 26–31), but the largest of these only included 36 esophageal adenocarcinoma cases and 34 Barrett's esophagus cases (13). Consistent with these prior smaller studies, we also observed increased expression of nine miRNAs in the cancer cases [miR-21 (8, 9, 13), miR-93 (8), miR-409-3p, miR-424 (13), miR-196a, miR-196b (15), miR-125b, miR-197, and miR-513 (14)]. This is in contrast to two studies that did not identify any miRNAs with increased expression in esophageal adenocarcinoma (10, 11).

**Table 3.** Observed and potential mRNA targets and predicted altered KEGG pathways of miRNAs increased in esophageal adenocarcinoma compared with Barrett's esophagus

| | | Predicted mRNA targets | | |
|---|---|---|---|---|
| **miRNA** | **Validated mRNA targets** | **DIANA-microT-CDS[b]** | **TargetScan 6.2[c]** | **Predicted targeted KEGG pathways[a]** |
| miR-4286 | No validated mRNA targets | ATXN7L3, HDDC3, HGD, LRRC4, MAN1A2, MED1, NDST1, PRX, PSM11, RHEBL1 | ANKRD34A, FOXO4, LRRC4, NDST1, NSD1, PPIL1, RNF43, SEMA4F, UBE2R2, ZBTB7B | Glycosphingolipid biosynthesis, mucin type O-glycan biosynthesis, amoebiasis |
| miR-630 | BCL2 (42), IGF1R (43), SNAI2 (44), YAP1 (42) | CCDC71L, CNEP1R1, GAD2, HMGCR, LYPD6, SOCS2, SUB1, VSIG1, WDPCP, ZNF770 | ATP1A1, BCO2, BRPF3, CDKAL1, LMO3, MED13L, MEF2D, MFSD6L, PARP3, PRDM13 | Taurine and hypotaurine metabolism, steroid hormone biosynthesis, salmonella infection |
| miR-575 | No validated mRNA targets | CHTOP, DENND5A, HOXD3, KLH29, PSG5, RIPK4, RSBN1, TOX, TRAPPC10, ZNF726 | CADM2, CSTA, DEEND5A, FBW11, GCLC, KLHL29, RIPK4, SBNO1, SGCD, WDFY3 | Glycosaminoglycan biosynthesis, Melanoma, Spliceosome, Non-small cell lung cancer |
| miR-494[d] | BCL2L11 (45), CDK6 (46), c-Myc (47), PTEN (48), RAD23B (49) | ARHGAP5, CXXC4, CYSTM1, GIN1, LIF, PPARGC1A, RBM4B, SOCS6, ZC3H7A, ZNF207 | B3GALT2, DCP1B, DZIP3, EVI5, HMGCS1, LRRC19, MAP4, RBM4B, SLC25A40, SOCS6 | Phosphatidylinositol signaling system Glioma, inositol phosphate metabolism, p53 signaling pathway, mTOR signaling pathway, PI3K-Akt signaling pathway, Cancers: endometrial, melanoma, small cell lung, and prostate |
| miR-320e[e] | No validated mRNA targets | ADAMTS6, BPY2B, ELMOD3, IRF6, MAST4, MAT2A, PPP2R2C, SLC35G3, TRAPPC8, TMEM47 | ALX1, ANKRD28, CELF4, DCAF16, EPHA7, FBXO45, FN1, IRF6, RNF10, TCEAL8 | Prostate cancer, Glioma, HIF1 signaling pathway, Hepatitis B, "Pathways in cancer" |
| miR-663b | PIK3CD (50), WAF1/CIP1 (51) | CD28, CXXC4, E2F8, FAM161B, HIST1H4D, NCR3LG1, NICN1, RPS6KA1, TGM4, TTC7A | EPHA10, GBF1, HAP1, MAP7D1, NKPD1, SBK1, STAT5B, STXBP5L, ZNF282, ZNF436 | Glycan degradation, sphingolipid metabolism, endocytosis, ECM–receptor interaction |
| miR-421[d] | ACE2 (52), ATM (53), CBX7 (54), FOXO4 (55), RBMXL1 (56), SMAD4 (57) | AFF4, DYNLRB1, FAM592A, FNBP1L, ONECUT2, PAM, PPP1CC, SLC25A3, TOMM70A, ZCAN4 | AFF3, AFF4, AKR1D1, CASP3, CTDSPL2, MBD2, PPP1cc, PRCC, SLC25A3, STRN3 | Spliceosome, transcriptional misregulation in cancer, p53 signaling pathway |
| miR-502-5p[e] | TRAF2 (58) | AP2B1, CCDC129, FIGN, GSTM2, NEDD4, PRDX2, RAB1B, SKAP1, SRPK2, SYT9 | ACO2, CDKN1B, DIO2, DNAJC3, DONSON, EXTL2, NRAS, PABPC1, RAB1B, SEMA6A | Chronic myeloid leukemia, cholinergic synapse, small cell lung cancer |
| miR-1915-3p | No validated mRNA targets | ANKRD52, CSPP1, FAM22D, GJC3, KCNE1L, NCR3LG1, PBXIP1, PLG2, SLC8A2, TNFSF11 | ADCYAP1R1, ANKRD52, IPK1, MAFF, MGAT3, MICU1, PDE1B, TCN2, TFE3, WSCD1 | Glycosphingolipid biosynthesis, Hedgehog signaling pathway, antigen processing and presentation, melanogenesis, HTLV-I infection, calcium signaling pathway, lysine degradation |
| miR-601 | No validated mRNA targets | ALDH3A1, CUL3, FAM196A, MCOLN3, POU2F2, RASGRP2, RICTOR, SIRT1, SNN, SRSF7 | B3GNT9, CUL3, EEA1, KCNJ12, LHFPL2, PRKACB, SGK494, SIRT1, SNN, TEC | Amphetamine addiction, arrhythmogenic right ventricular cardiomyopathy, cocaine addiction, cholinergic synapse |

[a]DIANA miRPath v2.0 using DIANA-microT-CDS (threshold 0.8), KEGG pathways $P < 0.01$ ranked by FDR $P$ value.
[b]Top 10 genes ranked by miTG score within DIANA-microT-CDS.
[c]Top 10 ranked by context score.
[d]$P < 0.001$ Tarbase and [e]$P < 0.001$ DIANA-microT-CDS (more stringent $P$ value cutoffs to limit list of target pathways).



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Distinct from these prior studies, which together observed increased esophageal adenocarcinoma expression of 11 other miRNAs, we observed nominal decreased rather than increased expression of six of these miRNAs [miR-192 (8, 12, 15), miR-200c (8), miR-194 (9, 15), miR-200a (9, 14), miR-215 (12), miR-301b (13)] and no difference in expression for the five other miRNAs [miR-147 (12), miR-615-3p (12), miR-223 (13), miR-450b-5p (13), miR-542-3p (13)]. The NanoString assay we used did not test expression of miR-560 (12), miR-326 (12), miR-618 (13), miR-25 (13), and miR-101 (14). Our ability to identify 46 miRNAs increased in esophageal adenocarcinoma may reflect our sensitive digital miRNA detection method and statistical power afforded by our large sample size.

A subset of miRNAs is commonly dysregulated in many human cancers, and an miRNA signature of cancer is emerging. One hallmark of solid tumors is the overexpression of miR-21 (38), with evidence that a global signature of cancer may include overexpression of miR-155 (39), miR-222 (39), and miR-17 (39). Consistent with other cancers, we observed nominally increased expression of miR-21-5p (Supplementary Table S3: fold increase 1.2; $P = 0.04$), miR-155 (fold increase 1.20; $P = 3 \times 10^{-4}$), and miR-222 (fold increase 1.12; $P = 0.08$), and miR-17 was not measured in our assay.

All 46 miRNAs that discriminate esophageal adenocarcinoma from Barrett's esophagus in this study are newly identified as associated with esophageal adenocarcinoma. Of the 10 most significantly increased miRNAs (top five from each analysis), six have previously been associated with various other cancers (miR-630, miR-494, miR-663b, miR-421, miR-502-5p, and miR-601; Supplementary Table S8), and many have validated mRNA targets or target pathways of cancer. For example, miR-630, miR-494, and miR-421 each have multiple validated mRNA targets known to be tumor suppressors (SMAD4 and PTEN) or oncogenes (c-Myc and BCL2), suggesting that these miRNAs may have causal roles in carcinogenesis. Bioinformatic tools applied to our results identified potential targeted KEGG cancer pathways, including prostate cancer (miR-494), non–small cell lung cancer (miR-575), and p53 signaling pathway (miR-494). Also of interest is enrichment of the taurine metabolism pathway (miR-630), given that taurine is a major constituent of bile, and bile reflux is a putative source of lower esophageal damage leading to esophageal adenocarcinoma. At least 18 of the 27 miRNAs that positively discriminated esophageal adenocarcinoma from Barrett's esophagus target KEGG pathways that have previously been associated with cancer.

As a proof of principle, we performed an internal validation study using 5-fold cross-validation, for unbiased feature (miRNA) selection and prediction to assess the ability of the most significant miRNAs to discriminate esophageal adenocarcinoma from Barrett's esophagus (17). As further testament to the strength and robustness of the most significant miRNAs, the same 10 were selected—based on *a priori*–specified criteria—from each of the five folds of data. Combined, these 10 miRNAs provided high, unbiased estimates of sensitivity (98%) and specificity (95%) with an AUC of 0.92. Moreover, pairwise combinations of these ten miRNAs also provided robust classification (Supplementary Table S7). These provisional miRNA "signatures" require replication in diverse external populations. If validated, these miRNA signatures—coupled with cheaper esophageal sampling techniques (40)—may enable improvement in the cost-benefit ratio of current surveillance programs (41). Even if the combinations we present from our study are sub-optimal in other populations, we have identified many miRNAs that distinguish esophageal adenocarcinoma from Barrett's esophagus and provide a framework for developing diagnostic and possibly predictive biomarkers.

An important strength of our study is its relatively large sample size, enabling us to robustly assess a broad range of miRNAs. We utilized a state-of-the art technology to digitally quantitate miRNAs in FFPE tissue without the need for amplification. For statistical analysis, we used two distinct strategies—detectability and quantitation—which enabled assessment of the specificity, sensitivity, and classification ability of miRNAs to discriminate these two glandular tissues by miRNA expression patterns.

Limitations of our study include the case–control design in which all Barrett's esophagus and esophageal adenocarcinoma tissues were from separate patients, rather than samples from patients who progressed from Barrett's esophagus to esophageal adenocarcinoma. However, the low rate of progression of individuals in surveillance programs presents challenges for any robust prospective analysis. Limited clinical data were available, such as smoking or gastroesophageal reflux disease (GERD) status, which may alter miRNA expression and partially explain our results. Also, we only included Barrett's esophagus patients without dysplasia, thus our study does not have the ability to assess miRNA profiles across the spectrum of the natural history of this disease. Native stratified squamous epithelium was also not included as a comparison group because this was not the primary goal of the study and such tissue is not usually targeted or retained. Although we present unbiased estimates of the predictive capability of miRNAs using 5-fold cross-validation, independent (external) validation is needed to provide a more objective quantification of the true predictive performance. Finally, although our study of 800 miRNAs has provided for a broad comparison of these tissues, more costly next-generation sequencing would provide a more comprehensive assessment of miRNAs.

In sum, this study provides evidence that tissue miRNA profiles can distinguish the glandular epithelia of esophageal adenocarcinoma from Barrett's esophagus. We identified 46 miRNAs that positively discriminate this cancer from its preceding metaplasia and demonstrated high and unbiased AUC estimates, which emphasizes the potential predictive ability of these biomarkers. Future replication studies in diverse populations will be required to provide for a broad independent validation of these candidate biomarkers.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the NIH.

## Authors' Contributions

**Conception and design:** J. Drahos, K. Schwameis, L.D. Orzolek, P. Birner, P.R. Taylor, S.F. Schoppmann, M.B. Cook

**Development of methodology:** J. Drahos, K. Schwameis, M.B. Cook

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** K. Schwameis, L.D. Orzolek, S.F. Schoppmann

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J. Drahos, K. Schwameis, P.R. Taylor, R.M. Pfeiffer, M.B. Cook

**Writing, review, and/or revision of the manuscript:** J. Drahos, K. Schwameis, H. Hao, P. Birner, P.R. Taylor, R.M. Pfeiffer, S.F. Schoppmann, M.B. Cook

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** K. Schwameis, H. Hao, P. Birner

**Study supervision:** K. Schwameis, P. Birner, M.B. Cook

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## Grant Support

This work was supported by the Intramural Program of the NCI at the NIH and the Department of Health and Human Services.

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Received February 11, 2015; revised November 11, 2015; accepted November 13, 2015; published OnlineFirst November 24, 2015.

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