Published in final edited form as:

Clin Cancer Res. 2009 September 15; 15(18): 5744–5752. doi:10.1158/1078-0432.CCR-09-0385.

MicroRNA Expression Signatures in Barrett's Esophagus and Esophageal Adenocarcinoma

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Abstract

Purpose—Esophageal adenocarcinoma (EAC) is a highly aggressive malignancy that frequently develops from Barrett's esophagus (BE), a premalignant pathological change occurring in the lower end of esophagus. To identify BE patients at high risk of malignant transformation is essential to the prevention of EAC. Although microRNA (miRNA) expression signatures have been associated with the etiology and prognosis of several types of cancers, their roles in the development of EAC have not been extensively evaluated.

Experimental Design—In this study, we analyzed the expression patterns of 470 human miRNAs using Agilent miRNA microarray in 32 disease/normal-paired tissues from 16 patients diagnosed with BE of either low or high grade dysplasia, or EAC.

Results—Using unsupervised hierarchical clustering and class comparison analyses, we found that miRNA expression profiles in tissues of BE with high grade dysplasia were significantly different from their corresponding normal tissues. Similar findings were observed for EAC, but not for BE with low grade dysplasia. The expression patterns of selected miRNAs were further validated using quantitative reverse transcription real-time PCR in an independent set of 75 pairs of disease/normal tissues. Finally, we identified several miRNAs that were involved in the progressions from low grade dysplasia BE to EAC.

Conclusions—We showed that miRNAs were involved in the development and progression of EAC. The identified significant miRNAs may become potential targets for early detection, chemoprevention, and treatment of esophageal cancer.

Keywords

microRNA; Barrett's esophagus; Esophageal adenocarcinoma

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Conflict of interest declaration None of the authors of this manuscript has conflict of interest to disclose.

INTRODUCTION

Esophageal cancer is a highly aggressive malignancy and the sixth most common cause of cancer-associated deaths worldwide (1). In the United States, especially in Caucasian males, both the incidence and the mortality of esophageal cancer have been steadily increasing during the past several decades (2,3). Despite the wide application of radical esophagectomy and systemic chemoradiotherapy, the overall five-year survival rate of esophageal cancer remains under 20%, mainly due to the fact that a large proportion of patients are diagnosed at an advanced stage (2,4). Therefore, it is critical for the control and treatment of this dreaded malignancy to identify clinically applicable biomarkers for early detection and targeted prevention.

There are two major histological types of esophageal cancer, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). EAC is one of the fastest growing malignancies in the United States with a six-fold increased incidence in the past three decades (3,5,6). EAC is usually associated with symptomatic gastroesophageal reflux disease and frequently develops from Barrett's esophagus (BE), a condition of pathological metaplasia or dysplasia in which the epithelial cells in the lining esophageal mucosa are replaced by premalignant columnar epithelium (7,8). Patients with BE are at a 30- to 125-fold increased risk of developing EAC (5–7,9). The malignant transformation rate of BE varies, depending on the presence of dysplasia in BE tissues, and specific host factors such as race, gender, and environmental exposures (9,10). Benign Barrett's metaplasia without dysplasia or with low grade dysplasia (LGD) exhibit a much lower malignant transformation rate compared to those with high grade dysplasia (HGD) (8,11,12). There has been considerable debate regarding the follow-up and radical treatment of BE patients due to low conversion rate and low cost-effectiveness (13,14). This highlights the importance of developing novel biomarkers that may further stratify BE patients, which will allow for the selection of those with the highest risk for malignant progression to receive more aggressive therapies.

MicroRNAs (miRNAs) are a group of small non-coding RNA molecules that are involved in a wide spectrum of basic cellular activities through their negative regulations of gene expression (15–22). Moreover, miRNAs have also been extensively associated with the etiology and clinical outcome of many human cancers (23–25). Previous genome-wide studies have reported that miRNA expression signatures could be used in cancer risk prediction, early diagnosis, histological classification, and prognosis assessment (16,26–29). However, to date, there have been only a few studies on global microRNA expression profiling in esophageal cancers (30,31). To our knowledge, this is the first study to examine miRNA expression profiles using paired tissues of various stages of BE and EAC to evaluate the role of miRNAs in the development and progression of EAC in Caucasians.

MATERIALS AND METHODS

Tissue samples

A total of 91 pairs of disease tissues and adjacent non-cancerous normal tissues of the surrounding esophagus from 91 patients were included in this study, which consisted of 16 pairs for the initial miRNA microarray experiments and 75 pairs for the subsequent quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) validations. For the microarray experiments, the 16 patients consisted of five BE patients with LGD, five BE with HGD, and six EAC patients. For qRT-PCR validation, the 75 patients included 26 BE with LGD, 24 BE with HGD, and 25 EAC. All BE samples were snap frozen tissues obtained from the Division of Gastroenterology and Hepatology at the Mayo Clinic, Rochester, Minnesota (32). An objective standard was used to distinguish LGD and HGD (33). The collection of EAC and adjacent surrounding normal tissues were as previously described (34,35). Briefly,

all EAC tissues were snap frozen tumor specimens collected at the time of diagnostic or therapeutic endoscopic biopsy procedures through an approved tissue collection protocol at The University of Texas M. D. Anderson Cancer. Corresponding normal esophageal squamous mucosa tissue was obtained for each EAC tissue. All tumors were staged based on the system described in the sixth edition of the American Joint Commission on Cancer Atlas (36). All BE and EAC specimens were reviewed by at least one experienced gastrointestinal pathologist before total RNA extraction.

MiRNA microarray

Total RNAs including small RNAs were extracted from each tissue using the mirVana miRNA extraction kit (Ambion, Austin, TX) according to the manufacturer's protocol. Total RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The miRNA microarray experiments were performed at the Cancer Genomics Core Laboratory at M. D. Anderson Cancer Center, using the Agilent Human miRNA Microarray Kit version 1.0, including 470 human miRNAs, 17 Kaposi Sarcoma-associated herpesvirus miRNAs, 14 human cytomegalovirus, and 32 Epstein Bar virus miRNAs (Agilent Technology, Foster city, CA) (37). For each miRNA, multiple probes were spotted on the array and the average intensity of these probes was calculated to represent the expression value of the miRNA. In addition, multiple spots were included as negative controls. For each tissue sample, 100 ng total RNA was hybridized with the miRNA array and further processed in accordance with the manufacturer's instructions. The arrays were scanned using an Agilent Technology G2565BA scanner and the scanned images were processed using the Feature Extraction software package version 9.5 (Agilent Technology). Coefficient of variation (CV) within groups of replicate probes was used as a quality control measure to reflect the intra-array reproducibility. The range of CV for highest quality, acceptable quality, and failure was determined by Agilent as $\leq 8\%$, $8\% - 15\%$, and $> 15\%$, respectively. In our study, the average CV $\pm$ standard deviation (range) of all 32 arrays was $6.46\% \pm 1.17\%$ (5.10%-10.07%). Among the 32 arrays, 29 arrays had the highest quality with a CV below 8%. Three arrays had an acceptable quality with a CV between 8% and 15% (8.17%, 9.31%, and 10.07%).

Quantitative reverse transcription real-time PCR

To select the miRNAs for validation, we first compared our results with those of the study by Guo et al. that identified seven miRNAs through a step-wise selection procedure as the most relevant miRNAs differentiating tumor and normal esophageal tissues in ESCC (30). In that study, three (hsa-miR-25, hsa-miR-424, and hsa-miR-151) of the seven miRNAs exhibited an increased expression in tumor tissues whereas four (hsa-miR-100, hsa-miR-99a, hsa-miR-29c, and hsa-miR-140) showed a decrease. In our study, five of these seven miRNAs showed the same direction of expression change and had at least a borderline statistical significance (P values ranged from 0.0009 to 0.07, Supplementary Table 1). However, two miRNAs, hsa-miR-29c and hsa-miR-140, which showed a significantly reduced expression in the study of Guo et al. exhibited a significantly increased expression in our study (Supplementary Table 1). For the comparison and validation purpose, we conducted qRT-PCR to determine the expression level of four of the seven miRNAs in 25 pairs of esophageal tumor and adjacent normal tissues, including one of the three consistently up-regulated miRNA (hsa-miR-25), one of two consistently down-regulated miRNA (hsa-miR-100), and the two inconsistent miRNAs (hsa-miR-29c and hsa-miR-140). We also validated hsa-miR-146a which was up-regulated in both HGD and EAC tissues in our initial microarray experiments. *TaqMan* MicroRNA Assay (Applied Biosystems, Foster city, CA) was used to quantify miRNA expression with a protocol slightly modified from the one provided by the manufacturer. In brief, 100 ng of DNase I-treated total RNA was reverse-transcribed in 1 X RT buffer containing pooled stem-loop RT primers for all the miRNA genes and one endogenous control (5.6 nM each), dNTPs (each at 250 μ M), 0.6 U of RNase Inhibitor, and 50 U of MultiScribe Reverse Transcriptase (Applied

Biosystems). Reverse transcription reactions were incubated at 16 °C for 30 minutes, 42 °C for 45 minutes, and 85 °C for 5 minutes. Real-time PCR was performed in duplicates in 10 µl volume containing 0.4 µl of microRNA assay mix and 5 µl of *TaqMan* 2X PCR Master Mix. PCR conditions were 50 °C for 2 minutes, 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute. Expression data for miRNA were acquired and analyzed using ABI PRISM 7900HT Sequence Detection System and SDS 2.1 software (Applied Biosystems). Small RNA U48 was used as internal control for input normalization. The cycle number at which the real-time PCR reaction reached an arbitrarily determined threshold (CT) was recorded for both the miRNAs and U48, and the relative amount of miRNA to U48 was described as $2^{-\Delta CT}$ where $\Delta CT = (CT_{miRNA} - CT_{U48})$.

Statistical analyses

The microarray data was analyzed using the BRB-ArrayTools version 3.70, developed by Dr. Richard Simon and Amy Peng Lam (38). Data points flagged as absent or low-quality signals by the Feature Extraction software were removed from further analyses. Data points flagged as valid signals were $\log_2$ transformed after thresholded at 6.67, the average intensity of all negative control spots on the arrays. After $\log_2$ transformation, normalization was conducted using the median over entire array method and normalization to median array as reference. Gene filters were then applied to exclude a miRNA from all arrays if (1) less than 20% of the expression values of the miRNA had at least an 1.5-fold change in either direction from the median expression value, or (2) the miRNA had over 50% missing or filtered out data points. Unsupervised hierarchical clustering was carried out to generate the tree structures of both arrays and median-centered genes, using the Cluster 3.0 program using the average linkage clustering algorithm with centered correlation as similarity metric (39). The clustering results were visualized using the TreeView program (39). For each disease group (LGD, HGD, or EAC, determined by pathological review), differentially expressed miRNAs between disease and normal tissues were identified using the Significance Analysis of Microarrays (SAM) (40) with the global false discovery rate (FDR) controlled at 5%. The miRNAs showing significant differences in the ratio of disease/normal expression values between different progression stages were identified by unpaired student's t test implemented in the class comparison module in BRB-ArrayTools. The disease/normal intensity ratios were calculated by subtracting the value of $\log_2$ transformed normal tissue intensity from the $\log_2$ transformed disease tissue intensity. The derived *P* values were adjusted by 10,000 univariate permutation tests. For each gene, the permutation *P* value was calculated as a proportion of permutations for which the *P* value of the univariate test was less than the *P* value of the student's t test. The expression data for each miRNA of the qRT-PCR was calculated using paired student's t test. All *P* values reported in this study were two-sided.

RESULTS

MiRNA signatures discriminated between disease tissues and paired normal tissues

To identify miRNA signatures that are associated with EAC development, we performed miRNA microarray analysis on tissues of 16 Caucasian patients consisting of five BE with LGD, five BE with HGD, and six EAC patients (Table 1). A total of 170 miRNAs had a signal intensity level above the threshold value in at least 75% of samples, which was comparable to the percentage of reliably detectable miRNAs reported in previous studies (27,30). After applying gene filters to further screen out the miRNAs that were unlikely to be informative, we identified 111 miRNAs that remained in the final analyses. We used unsupervised hierarchical clustering to differentiate the disease tissues from their paired normal tissues (Figure 1). We first analyzed all the tissues together and found that these tissues clustered into two major groups with 16 tissues in each group. Most normal tissues and diseases tissues were separately grouped except for three normal tissues (LGD-N1, LGD-N2, HGD-N1) that were

grouped with other diseases tissues whereas three disease tissues (LGD-T1, LGD-T2, LGD-T3) were grouped with other normal tissues (Figure 1A). It was noted that all the misclassified disease tissues were LGD tissues, indicating that there might not be significant differences in terms of miRNA expression patterns between normal tissues and BE with LGD. To confirm these observations, we further performed unsupervised hierarchical clustering within each specific disease type (Figure 1B–1D). We found that four out of ten tissues were misclassified in the group of LGD and corresponding normal tissues (Figure 1B). In comparison, only one sample was misclassified in the HGD group (Figure 1C). In the EAC groups, the samples were classified with 100% accuracy between disease and normal tissues (Figure 1D).

Significantly differentially expressed miRNAs between disease tissues and paired normal tissues in different disease groups

The miRNAs that were differentially expressed between disease and paired normal tissues in each disease group, as well as their chromosome locations and host or overlapping genes, were summarized in Table 2. When the global FDR was controlled at 5%, we could not identify any such miRNA in the LGD group (data not shown). In comparison, we found 32 and 39 miRNAs for the group of HGD and EAC, respectively. Among these miRNAs, 24 miRNAs exhibited the same trend of expression change in both groups, including 14 miRNAs (hsa-miR-126, hsa-miR-143, hsa-miR-145, hsa-miR-146a, hsa-miR-181a, hsa-miR-181b, hsa-miR-195, hsa-miR-199a, hsa-miR-199a*, hsa-miR-199b, hsa-miR-28, hsa-miR-29c, hsa-miR-30a-5p, and hsa-miR-424) that were up-regulated in disease tissues and 10 miRNAs (hsa-miR-149, hsa-miR-203, hsa-miR-205, hsa-miR-210, hsa-miR-221, hsa-miR-27b, hsa-miR-494, hsa-miR-513, hsa-miR-617, and hsa-miR-99a) down-regulated in disease tissues. For nine (hsa-miR-126, hsa-miR-143, hsa-miR-145, hsa-miR-181a, hsa-miR-181b, hsa-miR-199a, hsa-miR-199a*, hsa-miR-28, hsa-miR-30a-5p) of the 14 up-regulated miRNAs, the ratio of expression level in disease tissues to that of normal tissues was higher in EAC than in HGD. For seven (hsa-miR-149, hsa-miR-203, hsa-miR-210, hsa-miR-27b, hsa-miR-513, hsa-miR-617, and hsa-miR-99a) of the 10 down-regulated miRNAs, the ratio of disease to normal expression level was lower in EAC than in HGD.

Validation by quantitative reverse transcription real-time PCR

Five miRNAs were validated using qRT-PCR in an independent set of 75 normal/disease paired tissues, including 26 BE with LGD, 24 BE with HGD, and 25 EAC. The replication results were consistent with those of our initial microarray experiments, and all *P* values were more significant (*P* values ranged from 0.00006 to 0.25) than those in the microarray experiments except for hsa-miR-29c (*P* = 0.25) (Table 3).

MiRNA signatures associated with the progression of EAC

Using the class comparison module implemented in the BRB-ArrayTools, we identified 11 significant miRNAs that may be important in the progression from LGD to HGD, five (hsa-miR-200a*, hsa-miR-513, hsa-miR-125b, hsa-miR-101, and hsa-miR-197) were up-regulated and six (hsa-miR-23b, hsa-miR-20b, hsa-miR-181b, hsa-miR-203, hsa-miR-193b, and hsa-miR-636) were down-regulated (Table 4). Seven miRNAs were potentially important in the progression from HGD to EAC, and all of them were down-regulated in EAC, including four members of the let-7 miRNA family (hsa-let-7b, hsa-let-7a, hsa-let-7c, hsa-let-7f), hsa-miR-345, hsa-miR-494, and hsa-miR-193a. All the *P* values remained significant after adjusted by 10,000 univariate permutation tests (Table 4).

DISCUSSION

In this study, we assessed the miRNA expression patterns in patients with different stages of Barrett's esophagus and esophageal adenocarcinoma. To our knowledge, this is the first study

using paired tissues of various stages of BE and EAC to evaluate the global miRNA expression patterns in the development and progression of esophageal adenocarcinoma. We found a large number of differentially expressed miRNAs between HGD and their paired normal tissues as well as between EAC and their paired normal tissues. Unsupervised hierarchical clustering using the complete set of 111 miRNAs differentiated the EAC tissues with 100% accuracy. In addition, we also observed that for a large majority of overlapped miRNAs in these two tissue groups, the disease/normal intensity fold changes were more dramatic with more significant *P* values in the EAC group (Table 2), suggesting that the oncogenic changes mediated by these miRNAs may be further intensified during the progression to EAC. In contrast, we could not identify any significantly differentially expressed miRNAs between LGD and paired normal tissues. These observations were in line with literature showing the indistinguishable pathological features between LGD and regenerative changes of normal esophageal cells, and the significantly lower malignant transformation rate in LGD compared to HGD (8,10–12, 41). Taken together, our data strongly indicate that miRNA may play a more prominent role in the progression from LGD to EAC than in the initial transformation from normal esophagus cells to LGD.

Guo et al. has evaluated the miRNA expression differences between normal esophagus tissues and ESCC (30). Using a step-wise elimination procedure, they identified a signature with a minimum set of seven miRNAs to differentiate normal and tumor tissues with the highest accuracy. Five of the seven miRNAs also showed the same direction of expression change in our microarray analysis with statistical significance (Supplementary Table 1). For the two miRNAs that exhibited an opposite direction of expression change, we confirmed our results in an additional set of 25 pairs of EAC samples. These findings were biologically plausible since some miRNAs may perform similar functions in the development of both EAC and ESCC, whereas other miRNAs may play distinctive roles in these two histologies. It is also possible that the differences are due to the racial disparity between Chinese and Caucasian populations. Further studies are warranted to compare the roles of miRNAs in these two subtypes of esophageal cancer.

In another recent report on miRNA expression in esophageal cancer, Feber et al. (31) used the mirVana miRNA Bioarrays (Ambion) to evaluate the expression of 287 human miRNAs in 31 esophageal specimens including 10 EAC, 10 ESCC, 5 BE without dysplasia, 1 HGD, and 9 normal squamous epithelium (NSE) tissues. Among the 13 human miRNAs that showed differential expression between EAC and NSE tissues, eight (hsa-miR-27b, hsa-miR-203, hsa-miR-205, hsa-let-7c, hsa-miR-342, hsa-miR-100, hsa-miR-21, and hsa-miR125b) passed the filtering criteria of our study and was included in the 111 miRNAs that remained within our final analysis. All of the eight miRNAs exhibited a consistent direction of expression change between the data of Feber's and ours (Supplementary Table 1, *P* values ranged from 0.00005 to 0.12), further supporting the robustness of our results.

We also identified miRNAs showing statistically significant differences between the different progression stages of EAC. Among the 11 miRNAs implicated in the progression from LGD to HGD, hsa-miR-200a* and hsa-miR-125b showed an over 13-fold and 9-fold increase in the disease/normal signal intensity ratio, respectively. Hsa-miR-200a* has not been associated with cancer; however, its complementary miRNA, hsa-miR-200a has been linked to the etiology and prognosis of many cancers including ESCC (30,42–44). Hsa-miR-125b was found to be up-regulated in colon cancers, but down-regulated in ovarian cancers, suggesting a cancer-specific functional pattern (43,45). Two miRNAs (hsa-miR-181b and hsa-miR-193b) had the most significant permutation-adjusted *P* value and both were down-regulated in HGD. This was consistent with the potential role of hsa-miR-181b as a tumor suppressor in brain tumorigenesis, but opposite to the increased expression of this miRNA in pancreatic and colon cancers (45–47), again suggesting a cancer-specific function. Another interesting finding was

that among the seven miRNAs showing significantly different disease/normal intensity ratios between HGD and EAC, four belong to the let-7 miRNA family, of which most members function as tumor suppressors through negative regulation of the *RAS* gene (48). Accordingly, *RAS* mutations and amplifications have been frequently identified in BE and EAC tissues (49,50). Taken together, the miRNAs identified in our progression signatures are biologically plausible and further functional dissections of these miRNAs and identification of their target genes are highly warranted.

A strength of our study was that all the patients were Caucasians and most (87.5%) were males, greatly reducing the confounding effects of race and gender. Although it should be cautioned that the limited sample size (16 pairs) in our initial microarray experiments could result in potential chance findings, we used strict statistical approaches to control for type I errors in each of the subsequent analyses. In addition, our microarray results are highly consistent with our real-time PCR validation, as well as with the results of two previous reports (30,31), indicating that the chances are small for our data to be false-positive. Nevertheless, our current results and additional significant miRNAs need to be further validated using larger-size prospectively collected patient samples before the clinical application of these biomarkers. Another limitation of our study was that we only compared miRNA expression patterns between tumor and normal tissues but did not relate these data to the clinical outcome of the EAC patients. Additional collection of clinical follow-up data and analyses of the correlations between miRNA signatures and patient outcome would shed more light into the functions of miRNAs not only in cancer development and progression, but also in treatment response and prognosis. Moreover, it will provide additional clinical significance if we include BE tissues without dysplasia or indefinite for dysplasia in future studies, because these are also major conditions frequently encountered in clinics.

In conclusion, we reported for the first time that miRNAs may play important roles in the development and progression of esophageal adenocarcinoma. The list of promising miRNAs identified in our study could provide potential therapeutic targets for early detection, chemoprevention, and treatment of esophageal cancer.

TRANSLATIONAL RELEVANCE

This is the first study to demonstrate that specific microRNA expression signatures are associated with the progression from Barrett's esophagus to esophageal adenocarcinoma. The validation and incorporation of the identified significant miRNAs with the currently available clinical variables may further stratify Barrett's esophagus patients, which will allow for the selection of those with the highest risk for malignant progression to receive targeted chemoprevention and more aggressive therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by National Cancer Institute grant CA111922, and a grant from The University of Texas M. D. Anderson Cancer Center, Dallas, Cantu, Park, and Smith families, Rivercreek Foundation.

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Figure 1.

Unsupervised hierarchical clustering of different tissue groups including (A) all tissue samples, (B) Barrett's esophagus with low grade dysplasia, (C) Barrett's esophagus with high grade dysplasia, and (D) esophageal adenocarcinoma. LGD-T: Barrett's esophagus with low grade dysplasia, LGD-N: normal paired tissue of LGD-T, HGD-T: Barrett's esophagus with high grade dysplasia, HGD-N: normal paired tissue of HGD-T, EAC-T: esophageal adenocarcinoma, EAC-N: normal paired tissue of EAC-T. Blue color: disease tissues clustered by unsupervised hierarchical clustering, yellow color: normal tissues clustered by unsupervised hierarchical clustering. Samples in red font are those misclassified by unsupervised hierarchical clustering. Gray squares represent missing or filtered out data points.

Table 1
Host Characteristics of the 16 Study Subjects in the MiRNA Microarray Experiments.

Sample pair*	ID of disease tissue	ID of normal tissue	Disease type	Grade	Age at diagnosis	Gender	Ethnicity	Smoking status
LGD1	2312 (LGD-T1)	2311 (LGD-N1)	Barrett's esophagus	Low grade dysplasia	61	Male	Caucasian	Current
LGD2	1782 (LGD-T2)	1781 (LGD-N2)	Barrett's esophagus	Low grade dysplasia	82	Male	Caucasian	Never
LGD3	1503 (LGD-T3)	1502 (LGD-N3)	Barrett's esophagus	Low grade dysplasia	75	Male	Caucasian	Former
LGD4	3437 (LGD-T4)	3436 (LGD-N4)	Barrett's esophagus	Low grade dysplasia	71	Male	Caucasian	Never
LGD5	3660 (LGD-T5)	3659 (LGD-N5)	Barrett's esophagus	Low grade dysplasia	58	Male	Caucasian	Former
HGD1	2732 (HGD-T1)	2731 (HGD-N1)	Barrett's esophagus	High grade dysplasia	62	Female	Caucasian	Former
HGD2	3495 (HGD-T2)	3494 (HGD-N2)	Barrett's esophagus	High grade dysplasia	79	Male	Caucasian	Former
HGD3	5307 (HGD-T3)	5305 (HGD-N3)	Barrett's esophagus	High grade dysplasia	80	Male	Caucasian	Former
HGD4	6470 (HGD-T4)	6469 (HGD-N4)	Barrett's esophagus	High grade dysplasia	74	Female	Caucasian	Never
HGD5	8887 (HGD-T5)	8885 (HGD-N5)	Barrett's esophagus	High grade dysplasia	50	Male	Caucasian	Former
EAC1	34686-3 (EAC-T1)	34686-8 (EAC-N1)	Esophageal adenocarcinoma	2	44	Male	Caucasian	Former
EAC2	40771-3 (EAC-T2)	40771-13 (EAC-N2)	Esophageal adenocarcinoma	2	72	Male	Caucasian	Former
EAC3	42743-7 (EAC-T3)	42743-12 (EAC-N3)	Esophageal adenocarcinoma	3	60	Male	Caucasian	Never
EAC4	38521-7 (EAC-T4)	38521-13 (EAC-N4)	Esophageal adenocarcinoma	3	44	Male	Caucasian	Current
EAC5	36141-1 (EAC-T5)	36141-7 (EAC-N5)	Esophageal adenocarcinoma	3	57	Male	Caucasian	Former
EAC6	40839-4 (EAC-T6)	40839-13 (EAC-N6)	Esophageal adenocarcinoma	3	78	Male	Caucasian	Never

* LGD-T: tissues of Barrett's esophagus with low grade dysplasia, LGD-N: paired normal tissue of LGD-T; HGD-T: tissues of Barrett's esophagus with high grade dysplasia, HGD-N: paired normal tissue of HGD-T; EAC-T: tissues of esophageal adenocarcinoma, EAC-N: paired normal tissue of EAC-T.

Table 2

Expressed MiRNAs between Disease and Normal Esophageal Tissues by Disease Groups *

HGD-T vs. HGD-N **				EAC-T vs. EAC-N **			miRNA characteristics	
HGD-T/HGD-N	P value §	FDR-adjusted P value	miRNA	Fold change (EAC-T/EAC-N)	P value §	FDR-adjusted P value	Chromosome location	Host or overlapping gene
0.001		0.02	hsa-miR-126	2.89	0.003	0.01	9q34	EGFL7, AGPAT2
0.04		0.10	hsa-miR-143	15.73	0.0005	0.005	5q32	NA ¶
0.04		0.10	hsa-miR-145	13.53	0.001	0.007	5q32	NA
0.02		0.06	hsa-miR-146a	2.61	0.04	0.08	5q33	NA
0.27 ‡			hsa-miR-149	0.22	0.04	0.07	2q37	GPCI
0.007		0.04	hsa-miR-181a	3.08	0.0003	0.004	1q31	NA
0.0005		0.02	hsa-miR-181b	2.76	0.002	0.008	1q31	NA
0.02		0.06	hsa-miR-195	3.29	0.007	0.02	17p13	C17orf49
0.09		0.16	hsa-miR-199a	10.72	0.003	0.01	19p13	DNM2
0.05		0.11	hsa-miR-199a *	8.21	0.003	0.01	19p13	DNM2
0.05		0.11	hsa-miR-199b	2.08	0.09 ‡		9q34	DNM1
0.01		0.05	hsa-miR-203	0.05	0.0003	0.004	14q32	NA
0.34		0.43	hsa-miR-205	0.06	0.12	0.17	1q32	NA
0.09		0.17	hsa-miR-210	0.17	0.0002	0.004	11p15	NA
0.05		0.11	hsa-miR-221	0.35	0.001	0.007	Xp11	NA
0.02		0.06	hsa-miR-27b	0.28	0.0009	0.007	9q22	C9orf3
0.01		0.05	hsa-miR-28	2.63	0.006	0.02	3q28	LPP
0.05		0.11	hsa-miR-29c	2.61	0.02	0.04	1q32	NA
0.02		0.06	hsa-miR-30a-5p	6.14	0.03	0.05	6q13	NA
0.02		0.06	hsa-miR-424	2.27	0.05	0.08	Xq26	NA
0.0009		0.02	hsa-miR-494	0.39	0.001	0.007	14q32	NA
0.002		0.03	hsa-miR-513	0.32	0.0005	0.005	Xq27	NA
0.007		0.04	hsa-miR-617	0.27	0.00001	0.002	12q21	LIN7A
0.09		0.16	hsa-miR-99a	0.13	0.0009	0.007	21q21	C21orf34, AP000473.2
0.18		0.28					17q21	HOXB4

HGD-T vs. HGD-N **			EAC-T vs. EAC-N **			miRNA characteristics		
HGD-T/HGD-N	P value §	FDR-adjusted P value	miRNA	Fold change (EAC-T/EAC-N)	P value §	FDR-adjusted P value	Chromosome location	Host or overlapping gene
0.12		0.21					17q11	ERALI
0.02		0.06					7p15	NA
0.02		0.07					Xp11	NA
0.01 [‡]							17q25	AATK
0.001		0.02					14q32	NA
0.08		0.15					17q11	ERAL
0.001		0.02					Xq27	NA
			hsa-let-7c	0.42	0.00005	0.002	21q21	C21orf34, AP000473.2
			hsa-miR-193b	0.33	0.002	0.01	16p13	NA
			hsa-miR-214	4.62	0.14	0.24	1q24	DNM3
			hsa-miR-219	0.44	0.06	0.12	6p21	SLC39A7
			hsa-miR-224	0.24	0.0000007 [‡]		Xq28	GABRE
			hsa-miR-23b	0.30	0.02	0.06	9q22	C9orf3
			hsa-miR-27a	0.47	0.02	0.06	19p13	NA
			hsa-miR-31	0.28	0.26	0.37	9p21	AL353732.14
			hsa-miR-33	0.43	0.009	0.04	22q13	SREBF2
			hsa-miR-422b [‡]	0.32	0.01	0.05	5q33	PPARGC1B
			hsa-miR-497	2.77	0.07	0.11	17p13	BCL6B, AC027763.2
			hsa-miR-557	5.89	0.004 [‡]		1q24	NA
			hsa-miR-630	4.20	0.003	0.01	15q24	NA
			hsa-miR-663	4.59	0.005	0.02	20p11	NA
			hsa-miR-7	6.33	0.19 [‡]		9q21	HNRPK

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identified in both the HGD-T/HGD-N and EAC-T/EAC-N groups. SAM analysis could not identify any significantly differentially expressed miRNA between

grade dysplasia, LGD-N; paired normal tissue of LGD-T; HGD-T; Barrett's esophagus with high grade dysplasia, HGD-N; paired normal tissue of HGD-T; EAC-T; paired normal tissue of EAC-T

the class comparison module of the BRB-ArrayTools.

[¶]NA, Not Available.

[‡]hsa-miR-422b was replaced by hsa-miR-378 in the MiRNA Registry because they are from the same precursor.

[‡]P value of paired *t* test using the raw data of the array.

Validation of Microarray Experiments using Quantitative Reverse Transcription Real-Time PCR.

Tissue group *	Microarray experiments				Quantitative reverse transcription real-time PCR			
	Pair number (N)	Fold change (disease/normal)	P value **	Change in disease tissue	Pair number (N)	Fold change (disease/normal)	P value **	Change in disease tissue
EAC-T/EAC-N	6	1.56	0.07	Up	25	3.42	0.01	Up
EAC-T/EAC-N	6	0.48	0.02	Down	25	0.60	0.005	Down
EAC-T/EAC-N	6	2.61	0.02	Up	25	1.69	0.25	Up
EAC-T/EAC-N	6	2.05	0.002	Up	25	2.55	0.00006	Up
EAC-T/EAC-N	6	2.61	0.04	Up	25	2.98	0.003	Up
HGD-T/HGD-N	5	4.54	0.02	Up	24	11.51	0.01	Up

include the results of the Barrett's esophagus with low grade dysplasia group since we could not identify any significantly differentially expressed miRNA in this group after multiple comparison; HGD-T: Barrett's esophagus with high grade dysplasia, HGD-N: paired normal tissue of HGD-T; EAC-T: esophageal adenocarcinoma, EAC-N: paired normal tissue of EAC-

ed t test

between the results of qRT-PCR and microarray experiments

Table 4

MiRNAs with Significantly Different Expression Patterns between Different Progression Stages from Barrett's Esophagus to Esophageal Adenocarcinoma.

miRNA	<i>P</i> value*	Permutation <i>P</i> value*	Fold change [§]
LGD-T to HGD-T[¶]			
hsa-miR-23b	0.007	0.02	0.69
hsa-miR-20b	0.01	0.02	0.64
hsa-miR-200a*	0.02	0.02	13.47
hsa-miR-181b	0.03	0.008	0.45
hsa-miR-203	0.03	0.03	0.67
hsa-miR-513	0.03	0.03	1.58
hsa-miR-193b	0.04	0.008	0.37
hsa-miR-125b	0.04	0.05	9.20
hsa-miR-101	0.04	0.05	1.83
hsa-miR-197	0.04	0.05	1.61
hsa-miR-636	0.04	0.03	0.24
HGD-T to EAC-T[¶]			
hsa-let-7b	0.009	0.009	0.63
hsa-let-7a	0.01	0.004	0.57
hsa-miR-345	0.02	0.02	0.50
hsa-let-7c	0.03	0.03	0.59
hsa-let-7f	0.03	0.009	0.59
hsa-miR-494	0.03	0.03	0.58
hsa-miR-193a	0.05	0.05	0.44

* *P* value of *t* test in the class comparison module of BRB-ArrayTools to compare the disease/normal signal intensity ratios between different disease groups

** *P* value adjusted after 10,000 univariate permutations

[§] Value of HGD-T/LGD-T or EAC-T/HGD-T

[¶] LGD-T: Barrett's esophagus with low grade dysplasia; HGD-T: Barrett's esophagus with high grade dysplasia; EAC-T: esophageal adenocarcinoma