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MicroRNA profiles of Barrett's esophagus and esophageal adenocarcinoma: Differences in glandular non-native epithelium

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Abstract

Background—The tissue-specificity and robustness of miRNAs may aid risk prediction in individuals diagnosed with Barrett's esophagus. As a initial step, we assessed whether miRNAs can positively distinguish esophageal adenocarcinoma (EA) from the precursor metaplasia Barrett's esophagus (BE).

Methods—In a case-control study of 150 EAs frequency-matched to 148 BE cases, we quantitated expression of 800 human miRNAs in FFPE tissue RNA using NanoString miRNAv2. We tested differences in detection by case group using the chi-square 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 five-fold cross-validation.

Results—We observed 46 distinct miRNAs significantly increased in EA compared with BE; 35 of which remained when restricted to T1b and T2 malignancies. Three miRNAs (miR-663b, miR-421, miR-502-5p) were detected in >80% EA, but <20% of BE. Seven miRNAs (miR-4286, miR-630, miR-575, miR-494, miR-320e, miR-4488, miR-4508) exhibited the most extreme differences in expression with >5 fold-increases. Using five-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 ten miRs which, together, provided 98% sensitivity and 95% specificity.

Conclusion—This study provides evidence that tissue miRNA profiles can discriminate EA from BE. This large analysis has identified miRNAs that merit further investigation in relation to pathogenesis and diagnosis of EA.

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Impact—These candidate miRNAs may provide a means for improved risk stratification and more cost-effective surveillance.

Introduction

Esophageal adenocarcinoma (EA) 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 U.S. and continues to rise (2, 3). Prevention and control of EA could be enhanced with improved risk prediction and early diagnosis. The precursor metaplasia, which precedes EA, is known as Barrett's esophagus (BE). BE increases the risk of EA 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.

MicroRNAs (miRNAs) represent a broad class of small RNA molecules, typically comprised of 18–22 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 EA, no study to date has quantitated a wide-range of miRNAs in >40 samples in an attempt to distinguish between the glandular epithelia of BE and EA (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 EA from BE.

Materials and Methods

Study population

The case population consisted of 150 individuals with pathologically confirmed EA. Cases were randomly selected from all patients receiving surgical resection of carcinomas of the esophageal 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 1992–2009, with macro-dissection 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 non-dysplastic BE patients at the same institution during 1992–2012 without EA using American College of Gastroenterology guidelines (16). BE patients frequency-matched on birth year (+/− 5 years) and sex to the EA case population. All selected BE 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 BE patient samples failed to provide adequate RNA material, leaving 148 BE and 150 EA 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 (EA or BE [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 EA cases and two 10 μm scrolls were collected for BE biopsies. Samples were sent to Johns Hopkins Medical Institute (JHMI) Deep Sequencing and Core Facility. The QIAGEN RNeasy FFPE kit was used to extract total RNA (Qiagen, Germantown, MD).

NanoString nCounter analysis

Total RNA samples were processed according to the manufacturer's protocol for the nCounter Human miRNA Expression Assay v2 kit (NanoString, Seattle, WA). 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 non-specific binding ("background noise") were measured by six negative-controls for each sample, the mean plus two standard deviations was 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 EA and BE tissues, we first assessed differences in detection vs non-detection in the raw data by calculating the proportion of individuals that expressed the miRNA above background noise. The chi-square test was used to compare proportions of detect/non-detect 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 BE and EA 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 five-fold cross-validation to obtain unbiased estimates of performance criteria for a model that predicted EA or BE 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 4 retained groups to select the top five miRNAs based on lowest *P*-values of those <6.25E–5 and more frequent detection in EA vs. BE, and the top five miRNAs from the quantitative analysis based on greatest increased median-fold expression in EA vs. BE with *P*-values <6.25E–5. We entered these selected 10 miRs 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 miRs selected from the quantitative analysis were modelled as log base 2 continuous. miRs 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 (miR) 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 analysis was performed using STATA version 13.0 (StataCorp LP, College Station, TX).

Predicted dysregulated mRNA targets and KEGG pathways

We used two web-based bioinformatics tools, DIANA-microT web-server v5.0 (18, 19) (based on Ensembl v69 & miRBase v18) and TargetScan 6.2 (20–23), to predict putative mRNA targets of the top five miRNAs that positively identified EA cases from BE in the detectability or quantitative analyses. We report potential mRNA targets from each of the two web-based bioinformatics 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 (24), 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 pathways with FDR corrected p-values of < 0.01 (24), except for miRNAs which 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 EA 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 (Supplemental Table 1). Of the 148 frequency-matched BE 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 EA cases compared with BE 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 BE 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 BE cases (miR-206, miR-600, miR-1305, miR-371a-5p) but had low-to-moderate expression in EA cases (13%–33% detectable).

In BE cases we observed 127 miRNAs with significantly greater detection rates compared with EA cases (Table 1 and Supplemental Table 2), the largest differences in detection of which were for miR-215 (59%), miR-574-3p (53%), miR-31-5p (53%).

Quantitative Differences of miRNA Expression

There were 24 miRNAs with a median expression ≥ 2-fold in EA tissues compared with BE tissues, the largest of which was a 34-fold increased median expression of miR-4286 (Table 2 and Supplemental Table 3). Six of the 24 miRNAs (miR-630, 575, 494, 320e, 4488, 4508) exhibited >5-fold median differences. In combination, Tables 1 and 2 provide 46 unique miRNAs that were higher in EA than BE cases.

There were 17 miRNAs that had a significantly greater median expression in BE tissues compared with EA tissues (Table 2 and Supplemental Table 4). A majority of these median fold-changes were in the range of 2–3. The largest increase, a 12-fold median expression in BE tissues compared with EA, was observed for miR-205-5p.

Results from both the detectability and quantitative analyses were unaffected when we excluded the 36% of EA cases that received neoadjuvant chemotherapy or chemoradiation (results not shown). Results were robust when the EA case group were restricted to T1b and T2 malignancies, with 35 of the 46 miRNAs remaining statistically significantly increased in EA when compared with BE (Supplemental Tables 5 and 6).

Discriminatory Ability of a miRNA Signature

From each of the five-fold cross-validation models, the same 10 miRs were repeatedly selected based on *a priori* specified criteria. These were miRs 663b, 421, 502-5p, 1915-3p, and 601 from the detectability analyses and miRs 4286, 630, 575, 494, and 320e from the quantitative analyses. A model including these ten miRs 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 0.73–0.96 (Supplemental Table 7).

Predicted mRNA Targets and Biological 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). Biological (KEGG) pathways predicted by DIANA miRPath v2.0 using the top 10 selected miRNAs that positively distinguished EA from BE are shown in Table 3. Specific cancers were identified with mRNAs targeted by 10–15 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 9–18 distinct miRNAs.

Discussion

We have elucidated a putative miRNA signature that appears to discriminate EA from BE. 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 46 distinct miRNAs increased in EA compared with BE, 35 of which were also statistically significantly increased when the EA case group was restricted to T1b and T2 malignancies. Notably, seven miRNAs (miR-4286, miR-630, miR-575, miR-494, miR-320e, miR-4488, miR-4508) positively discriminated EA with fold-increases > 5. Our results provide a broad catalog of altered miRNAs that merit further investigation in relation to the pathogenesis and diagnosis of EA.

Prior studies of miRNA signatures in BE and EA tissues have not provided consistent results (8–15, 26–31) [reviewed(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 EA from BE, as the miR 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 BE and EA (8–15, 26–31), but the largest of these only included 36 EA cases and 34 BE cases (13). Consistent with these prior smaller studies, we also observed increased expression of nine miRs 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 EA (10, 11).

Distinct from these prior studies, which together observed increased EA expression of 11 other miRs, we observed nominal decreased rather than increased expression of six of these miRs (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 miRs (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 EA 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 a 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 (Supplemental Table 3: fold-increase 1.2, p=0.04), miR-155 (fold-increase 1.20, p=3*10-4) and miR-222 (fold-increase 1.12, p=0.08); miR-17 was not measured in our assay.

All 46 miRs which discriminate EA from BE 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, miR-601; Supplemental Table 8) 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, PTEN) or oncogenes (c-Myc, BCL2), suggesting that these miRNAs may have causal roles in carcinogenesis. Bioinformatics 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 EA. At least 18 of the 27 miRNAs that positively discriminated EA from BE target KEGG pathways that have previously been associated with cancer.

As a proof of principle, we performed an internal validation study using five-fold cross-validation, for unbiased feature (miR) selection and prediction to assess the ability of the most significant miRNAs to discriminate EA from BE. (17) As further testament to the strength and robustness of the most significant miRs, the same ten were selected—based on *a priori* specified criteria—from each of the five folds of data. Combined, these ten miRs provided high, unbiased estimates of sensitivity (98%) and specificity (95%) with an AUC of 0.92. Moreover, pairwise combinations of these ten miRs also provided robust classification (Supplemental Table 7). 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 which distinguish EA from BE 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 BE and EA tissues were from separate patients, rather than samples from patients who progressed from BE to EA. However, the low rate of progression of individuals in surveillance programs presents challenges for any robust prospective analysis. Limited clinical data was available, such as smoking or GERD status, which may alter miRNA expression and partially explain our results. Also, we only included BE 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 miRs using five-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 EA from BE. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

miRNAs differentially detected among esophageal adenocarcinoma and Barrett's esophagus[1]

		Samples with Detectable Expression[2]			Median expression (p25, p75)[3]	
	miRNA: EA > BE	**EA (%)**	**BE (%)**	**p-value[4]**	**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)

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		Samples with Detectable Expression[2]			Median expression (p25, p75)[3]	
	miRNA: BE > EA[1]	EA (%)	BE (%)	p-value[4]	EA	BE
	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)

[1] Displaying top 25 of 127;

[2] miRNA detected above mean + 2 standard deviations of negative controls for individual samples;

[3] Normalized data with miRNA expression at or below background set to missing;

[4] Ordered by p-value

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[#] Listed in high confidence miRNA data set [24]

Table 2

miRNAs differentially expressed among esophageal adenocarcinoma and Barrett's esophagus

		Samples with Detectable Expression[1]		Median expression (p25, p75)[2]		p-value	Median Fold-Change[3]
	miRNA: EA > BE	EA (%)	BE (%)	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

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		Samples with Detectable Expression[1]		Median expression (p25, p75)[2]		p-value	Median Fold-Change[3]
	miRNA: BE > EA	EA (%)	BE (%)	EA	BE		
	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

[#] Listed in high confidence miRNA data set [24]

[1] miRNA detected above mean + 2 standard deviations of negative controls for individual samples;

[2] Normalized data with miRNA expression at or below background set to missing;

[3] Ordered by median fold-change

Table 3

Observed and potential mRNA targets and predicted altered KEGG pathways of miRNAs increased in EA compared with BE

miRNA	Validated mRNA Targets	Predicted mRNA Targets		Predicted Targeted KEGG Pathways[3]
		DIANA-microT-CDS[1]	TargetScan 6.2[2]	
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*	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**	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, HIF-1 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*	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**	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

[1] Top 10 genes ranked by miTG score within DIANA-microT-CDS

[2] Top 10 ranked by context score

[3] DIANA miRPath v2.0 using DIANA-microT-CDS (threshold 0.8), KEGG pathways p<0.01 ranked by FDR *p*-value

* *P*<0.001 Tarbase,

** *P*<0.001 DIANA-microT-CDS (more stringent p-value cut-offs to limit list of target pathways