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Circulating serum exosomal miRNAs as potential biomarkers for esophageal adenocarcinoma

Short title: Exosomal miRNAs and esophageal adenocarcinoma

Karen Chiam[1]

Tingting Wang[1]

David I Watson[1]

George C Mayne[1]

Tanya S Irvine[1]

Tim Bright[1]

Lorelle Smith[1,5]

Imogen A White[2]

Joanne M Bowen[3]

Dorothy Keefe[4]

Sarah K Thompson[5]

Michael E Jones[6]

Damian J Hussey[1]

Flinders University Department of Surgery[1], University of Adelaide School of Medicine[2], University of Adelaide School of Medical Sciences[3], Royal Adelaide Hospital Cancer Centre[4], University of Adelaide Discipline of Surgery[5], Flinders University Department of Anaesthesia and Pain Medicine, and Department of Anatomy[6], Adelaide, South Australia, Australia.

Author for Correspondence:

Dr Damian Hussey

Flinders University Department of Surgery

Room 3D211, Flinders Medical Centre

Bedford Park 5042, South Australia

Tel : 61-8-82046091

Fax : 61-8-2046130

email : damian.hussey@flinders.edu.au

Abstract

BACKGROUND: The poor prognosis and rising incidence of esophageal adenocarcinoma highlight the need for improved detection methods. The potential for circulating miRNAs as biomarkers in other cancers has been shown, but circulating miRNAs have not been well characterized in esophageal adenocarcinoma. We investigated whether circulating exosomal miRNAs have potential to discriminate individuals with esophageal adenocarcinoma from healthy controls and non-dysplastic Barrett's esophagus.

METHODS: 754 miRNAs were profiled in serum circulating exosomes from a cohort of 19 healthy controls, 10 individuals with Barrett's esophagus and 18 individuals with locally advanced esophageal adenocarcinoma. MiRNA expression was assessed using all possible permutations of miRNA ratios per individual.

RESULTS: 408 miRNA ratios were differentially expressed in individuals with cancer compared to controls and Barrett's esophagus (Mann Whitney U test, $P<0.05$). 179/408 ratios discriminated esophageal adenocarcinoma from healthy controls and Barrett's esophagus (linear regression, $P<0.05$; Area under ROC>0.7, $P<0.05$). A multi-biomarker panel (RNU6-1/ miR-16-5p, miR-25-3p/ miR-320a, let-7e-5p/ miR-15b-5p, miR-30a-5p/ miR-324-5p, miR-17-5p/ miR-194-5p) demonstrated enhanced specificity and sensitivity (Area under ROC=0.99, 95%CI 0.96-1.0) over single miRNA ratios to distinguish esophageal adenocarcinoma from controls and Barrett's esophagus.

CONCLUSIONS: This study highlights the potential for serum exosomal miRNAs as biomarkers for the detection of esophageal adenocarcinoma.

Keywords: Esophageal cancer, Barrett's Esophagus, Biomarkers, microRNAs, Exosomes, Serum

INTRODUCTION

Esophageal adenocarcinoma is one of the deadliest cancers, with an overall mortality rate of 85-90%. Its incidence has increased 6-fold over the past 3-4 decades [1, 2]. As outcomes are better when this cancer is treated early, endoscopy surveillance of individuals with the precursor Barrett's esophagus is undertaken to identify individuals who might benefit from early intervention. However, the annual rate of progression to cancer is reported to range from 0.12 to 0.4% [3], so the cost effectiveness of Barrett's esophagus surveillance has been questioned [4]. If less invasive detection tools such as a reliable blood test can be developed, then it might be possible to reduce dependence on endoscopy surveillance in high risk individuals, or more cost effective surveillance strategies might be developed.

MicroRNAs (miRNAs) are small non-coding 21-23 nucleotide RNA molecules, which regulate the production of proteins from mRNA [5, 6]. They are found in the cell free fraction of blood, including serum, and can be reliably measured from frozen stored samples [7]. Circulating miRNAs levels have recently been used to identify some cancers [8, 9]. miRNAs circulating in serum are present in a variety of forms, including within small extracellular microvesicles known as exosomes. Exosomes are shed by tumor cells, and can enter the circulation. Significantly, circulating miRNAs in exosomes are more stable than other forms, as they are protected from endogenous RNase degradation [10, 11], and have significant potential as disease specific biomarkers.

Unique circulating exosomal miRNAs signatures have been recently identified in colon cancer [12]. Robust studies evaluating circulating exosomal miRNAs signatures

in esophageal cancer are yet to be reported, with the only recent studies limited to either esophageal squamous cell carcinoma cell lines, or a very small clinical cohort (eight individuals with esophageal adenocarcinoma vs. four controls) in which a broad discovery approach was not applied [13-15]. Hence, to evaluate the potential for circulating miRNAs in exosomes to be used as biomarkers for esophageal adenocarcinoma, we applied a broader discovery approach to investigate levels of circulating exosomal miRNAs in serum from individuals with esophageal adenocarcinoma, compared to non-dysplastic Barrett's esophagus and age-matched controls. This approach was used to determine the potential for circulating miRNAs in exosomes to be used to identify individuals with esophageal adenocarcinoma.

MATERIALS AND METHODS

Patient recruitment

In our institution, individuals undergoing upper gastrointestinal endoscopy at Flinders Medical Centre in Adelaide, South Australia, were recruited to a research study that included collection and storage of blood samples for biomarker research. Other individuals presenting to the Royal Adelaide Hospital, Adelaide, South Australia, for investigation and treatment of esophageal cancer were also recruited to a parallel study that included collection of blood samples for biomarker research. Blood samples from 47 individuals who met the following criteria were selected for the current study (Figure 1):

i) Healthy **controls** (n=19). These individuals had blood collection prior to an endoscopy that was unrelated to investigation of cancer, Barrett's esophagus or gastroesophageal reflux. At endoscopy no abnormality was identified, and the presence of Barrett's esophagus, upper gastrointestinal cancer or gastroesophageal reflux was excluded.

ii) Individuals diagnosed with **non-dysplastic Barrett's esophagus** (n=10). These individuals had blood collection prior to endoscopy, Barrett's esophagus >2cm in length (median 4cm ± 1.5) was visualized at the endoscopy, and Barrett's esophagus was confirmed by mucosal biopsy that showed histopathological evidence of intestinal metaplasia but no dysplasia.

iii) Individuals diagnosed with **locally advanced esophageal adenocarcinoma** (n=18). These individuals also had blood collection prior to endoscopy at Flinders Medical Centre or imaging investigation at the Royal Adelaide Hospital. Esophageal adenocarcinoma was confirmed by histopathology, and staging investigations (CT scan, PET scan and endoscopic Ultrasound)

confirmed clinical T staging of T3 or higher in all instances. All bloods collected from these individuals were taken before any treatment (either surgery and/or chemoradiotherapy). 14 of these individuals were staged after surgical resection (T3, n=13; T4, n=1), and 4 had metastatic disease and did not undergo surgery for their advanced cancer (all T3 or T4).

As esophageal adenocarcinoma is associated with a strong male gender bias of up to 8:1 [16], all individuals contributing blood samples for this study were male. In addition the Barrett's esophagus and control groups were age matched to the cancer group with median age 70 ± 13, 65 ± 14 and 66 ± 10 respectively ($p=0.25$, ANOVA).

The recruitment and blood collection was approved by the Southern Adelaide Clinical Human Research Ethics Committee and the Royal Adelaide Hospital Research Ethics Committee, and all individuals gave written informed consent for blood and data collection and use in this study. The research work was conducted in accordance with the Declaration of Helsinki's (2008) Statement of ethical principles for medical research involving human subjects.

Blood processing, serum exosome isolation and Exosome miRNA extraction

Blood was collected in a serum clot activator tube and then processed by centrifugation. Centrifugation was performed at 400g for 15min, and 1ml aliquots of serum were stored in eppendorf tubes at -80°C for later use. Serum aliquots from individuals selected for this study were retrieved from storage, quick thawed, and centrifuged again at 16,000g at 4°C for 30min to remove large microparticles. To isolate exosomes, 250µl of supernatant was processed with ExoQuick^{TM} (System Biosciences, EXOQ20A-1) according to the manufacturer's protocol. All serum

samples were incubated with ExoQuick^{TM} for 16h. The acquired exosome pellet was resuspended in 50µl phosphate buffered saline (PBS). The presence of particles consistent in size with exosomes (60-150nm) was confirmed using a Nanosight LM10 Nanoparticle Analysis System and Nanoparticle Tracking Analysis Software (Nanosight Ltd).

Extraction of miRNA from exosomes was then performed using the commercial miRNeasy Serum/Plasma kit (Qiagen, #217184) according to the manufacturer's protocol. All RNA elution steps were carried out at 10,000g for 15s. Final RNA elution was performed with 24µl of RNase-free ultra pure water per sample.

TaqMan OpenArray miRNA profiling

The high throughput TaqMan® OpenArray® Human microRNA panel (Life technologies, #4461104) was used for miRNA profiling. This panel comprised 754 human miRNAs probes that are based on miRNA sequences derived from Sanger miRBase v14. For each sample, 3µl of RNA was reverse transcribed using pre-defined RT-primers (Megaplex^{TM} Primer Human Pool A and Pool B) and the TaqMan® microRNA Reverse Transcription Kit (Life technologies, #4366596). Pre-amplifications were carried out with Megaplex^{TM} PreAmp Pools and TaqMan PreAmp Master Mix on 7.5µl cDNA/ sample for each pool. The pre-amplified products (4µl per sample) were diluted at the recommended 1:40 dilution with 156µl of RNase-free ultra pure water before loading onto the 384-well TaqMan OpenArray loading plate. PCR runs were performed using the Biotrove OpenArray NT cycler at Flinders Genomics Facility, Flinders University.

Expression and statistical analyses

The cycle threshold (Ct) value per assay was assessed using the RealTime PCR Statminer® software analysis program (v4.5, Integromics). Integrity of the Cts obtained with these settings was checked on a subset of samples by manually assessing the amplification via the raw fluorescent signals. Only miRNAs with detectable Cts in all samples were considered for the expression analysis. The relative expression of each detectable miRNA was calculated as $2^{(40\text{-}Ct)}$. Relative expression values for each miRNA were used to derive per patient values for every possible permutation of miRNA ratios. Open Array™ data were deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo; GEO accession number GSE63108).

Significant miRNA expression differences were determined using Mann-Whitney U test (P<0.05) (R version 3.0.2). Prediction performance of each miRNA ratio candidate was assessed using linear regression (R version 3.0.2) and Receiver Operating Characteristic (ROC) curve analysis (SPSS v22, Inc). Unsupervised clustering was used to generate a heatmap of the miRNA signature using Genepattern version 3.9 (Broad Institute). Stepwise multiple linear regression was used to generate a potential multiple biomarkers panel (SPSS v22, Inc). The sequential statistical approach to identify potential biomarker candidates and the multiple biomarkers panel was checked by leave-one-out cross validation (R version 3.0.2).

RESULTS

Expression profiling of circulating miRNAs to identify a miRNA ratio signature in esophageal adenocarcinoma

Circulating serum exosomal miRNAs were profiled using the high throughput Taqman OpenArray qPCR platform. 100 miRNAs had detectable Cts in all 47 individuals. To identify reliable and reproducible miRNA biomarkers, expression analysis was confined to these miRNAs. MiRNA expression data values were then derived for all possible permutations of miRNA ratios within each individual. This ratio method was identical to that utilized by Boeri et al [17] in an analysis of data from individuals with lung cancer. Altogether 9900 miRNA ratios were obtained. Selection of the miRNA ratios with potential diagnostic value was performed using sequential testing as described in Figure 1. 408/9900 miRNA ratios were commonly differentially expressed in esophageal adenocarcinoma compared to healthy controls and in esophageal adenocarcinoma compared to non-dysplastic Barrett's esophagus (Mann-Whitney U test, $P<0.05$). This first subset of 408 miRNA ratios was then assessed by both linear regression and ROC curve analysis to determine their prediction performance for discriminating patients with esophageal adenocarcinoma from healthy controls and those with non-dysplastic Barrett's esophagus. 179/408 miRNA ratios were identified to have potential diagnostic value by linear regression analysis ($P<0.05$) and ROC (AUROC>0.7, $P<0.05$). This list of 179 miRNA ratios consisted of 82 highly expressed miRNA ratios, and 97 lowly expressed miRNA ratios, in individuals with esophageal adenocarcinoma compared to both healthy controls and Barrett's esophagus (Figure 2).

Potential benefit of combining multiple miRNA ratios into a biomarker panel

To investigate the potential of combining multiple miRNA ratios and compare its performance to a single miRNA ratio, stepwise multiple linear regression analysis was performed on the 179 miRNA ratios. Five miRNA ratios derived from 10 unique markers, RNU6-1/ miR-16-5p, miR-25-3p/ miR-320a, let-7e-5p/ miR-15b-5p, miR-30a-5p/ miR-324-5p and miR-17-5p/ miR-194-5p, were determined to be a potential multiple biomarkers panel that could discriminate individuals with esophageal adenocarcinoma from controls and Barrett's esophagus (Table 1). These 5 miRNA ratios were identified to be significant independent biomarkers (standardized regression coefficients, $P<0.05$). Subsequent inclusion of each of the ratios contributed significantly to the model, as demonstrated by significant F change of increase in adjusted R^2, with a final adjusted R^2 of 0.70 (Table 1). The distribution and normality plots of the standardized residuals for the multiple biomarkers model did not suggest any abnormality or outliers (Supplementary Figure 1). The validity of the full sequential statistical methods used in this study, from the initial biomarkers selection (Mann-whitney, linear regression and ROC analyses) to the modeling of biomarkers combination with stepwise multiple linear regression, were confirmed by leave-one-out cross validation (Supplementary Table 1).

3/5 ratios were significantly elevated in esophageal adenocarcinoma whereas 2/5 ratios were decreased in esophageal adenocarcinoma compared to controls and Barrett's esophagus (Table 2). RNU6-1/ miR-16-5p was the most highly expressed miRNA ratio in cancers, with 3.7 fold and 3.8 fold difference compared to controls and Barrett's esophagus respectively. While miR-30a-5p/ miR-324-5p was the most decreased miRNA ratio in cancers compared to controls (3-fold difference) and

Barrett's esophagus (1.6 fold difference). The expression of each of the 5 miRNA ratios were comparable between controls and Barrett's esophagus (Supplementary Figure 2).

Prediction performance of the 5-miRNA ratios combination compared to single miRNA ratios

The prediction accuracy of the 5 miRNA ratios as independent biomarkers and as a combined biomarkers panel were evaluated by ROC curve analysis. The single best performing biomarker was the top highly expressed miRNA ratio in esophageal adenocarcinoma, RNU6-1/ miR-16-5p, with an AUROC of 0.89 (95% CI 0.79-1.00) (Figure 3). The combination of all the 5 miRNA ratios significantly enhanced the prediction accuracy to an overall AUROC of 0.99 (95% CI 0.96-1.00) (Figure 3).

To further investigate how each of the 5 miRNA ratios would add value to the biomarker panel, the accuracy of predicting cancer in a patient was assessed per addition of each miRNA ratio (Table 3). RNU6-1/ miR-16-5p as a single biomarker predicted 14/18 cancer patients (78% accuracy). Stepwise inclusion of the remaining 4 miRNA ratios to RNU6-1/miR-16-5p in the panel demonstrated improved prediction accuracy, up to 94% prediction accuracy when all 5 miRNA ratios were included. The AUROC increased at each inclusion step, further supporting that each ratio added to the panel provided some prediction value. The improvement of prediction accuracy by adding more than 1 miRNA ratio in the biomarker panel was further supported by the leave-on-out cross validation (Supplementary Figure 3).

DISCUSSION

To our knowledge, this is the first study to comprehensively profile and compare circulating exosomal serum miRNAs in esophageal adenocarcinoma, non-dysplastic Barrett's esophagus and healthy controls. We identified a miRNA signature of 179 miRNA ratios and a multiple biomarkers panel of 5 miRNA ratios, which have potential diagnostic value for esophageal adenocarcinoma. The study was designed as the initial step towards developing clinically applicable diagnostic biomarkers. The results of our study support further investigation in expanded cohorts, and within an expanded range of disease cohorts.

In our current study, the 5-miRNA ratios biomarker panel consisted of several miRNAs known to be associated in cancers as either oncogenes or tumor suppressors. Notably, 5/9 of the miRNAs in the multiple biomarkers panel have been reported previously to be differentially expressed between Barrett's esophagus and esophageal adenocarcinoma in human tissues [18-20]. In a Taqman miRNA profiling study on human tissues, Wu et al found that miR-25-3p is progressively overexpressed from normal esophageal epithelium to non-dysplastic Barrett's esophagus, dysplastic Barrett's esophagus, and to esophageal adenocarcinoma [19]. A small tissue biomarker pilot study also indicated that miR-15b-5p expression alone discriminated non-dysplastic Barrett's esophagus from dysplastic Barrett's esophagus and esophageal adenocarcinoma (87% sensitivity and 80% specificity) [18]. Importantly, comparison between our current serum based study and a previous study undertaken in our laboratory which evaluated tissue based miRNAs associated with development of esophageal adenocarcinoma identified an overlap of 4 miRNAs, miR-194-5p, miR-30a-5p, miR-15b-5p and miR-17-5p [20]. miR-194-5p and miR-30a-5p have been

shown to be associated with epithelial-mesenchymal transition, which is one of the hallmarks of more aggressive cancers [21, 22]. Recent studies have demonstrated an important role for miR-15b-5p (and miR-16-5p that lies in the same genomic cluster), in regulating apoptosis, cell cycle and DNA repair pathways [23], and highlighted miR-15b-5p as a potential target for anti-cancer therapies [24]. Finally, miR-17-5p belongs to one of the most well-studied oncogenic miRNA clusters, miR-17/92, commonly found to be overexpressed in solid tumors including gastric and colorectal cancers [25]. The miR-17/92 cluster family of miRNAs has been shown to target universal cancer genes such as PTEN and E2F which are critical for regulating cell growth and cell death [25]. Altogether, these findings suggest that changes in the expression of tissue miRNAs with biological roles in the malignant degeneration of esophageal tissue may be reflected in the serum exosomes of the individuals.

The ratio method used in our study was the same as that utilized by Boeri et al to develop diagnostic and prognostic miRNA signatures for lung cancer [17]. Boeri et al recently validated their miRNA signature in a large cohort of individuals with lung cancer [26], highlighting the potential for application in other cancers. We adopted this method to overcome the limitations associated with global normalization approaches. Reliable normalization is absolutely vital for an accurate assessment of circulating miRNA data. However, this remains one of the main challenges for studies on expression analysis of circulating miRNAs due to a lack of established universal housekeeping genes and standardized approach for serum samples [27-29]. Furthermore, while global normalization may be deemed as the currently more acceptable method for large miRNA datasets, it is not realistic to implement its usage in the clinical setting. Currently, the common housekeeping genes include small

nuclear/nucleolar RNAs such as RNU44, RNU48 and RNU6-1, also more commonly known as U6. The spliceosome complex, which catalyzes pre-mRNA splicing, and in which U6 is a key functional component, is frequently deregulated in a wide variety of cancers [30]. Consistent with this and with our findings, it has been shown that serum RNU6-1 levels are differentially expressed in serum from individuals with breast cancer compared to healthy controls [31]. In addition, a recent miRNA profiling study performed on the serum exosomes in individuals with glioblastoma identified RNU6-1 as a potential diagnostic cancer biomarker [32]. Thus, at least in the context of serum miRNA profiling studies in cancers, we propose that RNU6-1 is not suitable for use as a normalization control gene, and alternative methods of analysis are required.

A strength of our current study, at least from a clinical translation perspective, is the use of serum rather than tissue biomarkers. A significant issue with biomarker studies that utilize esophageal adenocarcinoma cancer tissues is heterogeneity within individual cancers. This can impact on the reproducibility of study outcomes, and hence translation to clinical practice. Heterogeneity generates sampling issues, as different patterns of expression can be identified in different samples from different parts of the same tumor from the same individual. This is a particular problem when studies use small endoscopic biopsies, as these are inevitably collected from different parts of the tumor. Blood and serum derived biomarkers, however, offer an opportunity to overcome this sampling problem, and serum derived diagnostic biomarkers miRNAs for esophageal adenocarcinoma, if appropriately validated, might be more robust and therefore more readily translated into a clinical useful blood-based test.

There are limitations to our current study, and more work is required to investigate the specificity and sensitivity of serum exosomal microRNAs for esophageal adenocarcinoma. In this study, all individuals with cancer had locally advanced disease, and we did not evaluate serum from individuals with early stage cancer (T1) or high grade dysplasia in Barrett's esophagus. If this approach and the current biomarker panel can be used to distinguish individuals with very early stage cancer from those with Barrett's esophagus then the serum biomarker panel could be used for surveillance of individuals with Barrett's esophagus, and new paradigms for surveillance could be developed which reduce the need for endoscopy. However, more work is required using serum from cohorts with early esophageal adenocarcinoma and high grade dysplasia in Barrett's esophagus to address this question. In addition, our study has only examined esophageal adenocarcinoma, and not other cancer types, so the biomarker panel must also be tested against other cancers, including gastrointestinal cancers and other cancer types, to determine its specificity to esophageal adenocarcinoma. However, the outcomes of the current study do demonstrate significant potential for serum exosomal miRNAs to be developed for cancer diagnosis, and more work to address this is certainly justified.

The potential advantage of combining multiple biomarkers vs. the use of single biomarkers alone was demonstrated in this study. However, due to the small cohort size, we were unable to fully investigate other factors required in developing a multi-biomarkers panel. For instance, we were unable to use our current cohort to determine the appropriate number of biomarkers that can be included in a single panel. This would require a larger cohort with the extended disease groups mentioned above.

In conclusion, our study has demonstrated that circulating exosomal miRNAs have potential to act as diagnostic biomarkers for esophageal adenocarcinoma, and combining miRNAs might improve the sensitivity and specificity of this approach. However, the limited size of the cohort studied, and the need to test the markers in other clinical cohorts means that more work is required before considering broader clinical application. We anticipate that our study's findings will encourage future studies to investigate circulating miRNAs for clinical utility in esophageal adenocarcinoma and other cancers.

Author contributions

All authors participated in aspects of study design, data acquisition and analysis, and preparation of the manuscript.

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Table 1: Stepwise multiple regression model of the multiple biomarkers panel of 5 miRNA ratios that discriminated individuals with esophageal adenocarcinoma from healthy controls and Barrett's esophagus. The order of display of each miRNA ratio refers to the order of their stepwise inclusion in the regression model.

Model	**Standardized Coefficients**			**R**	**R-square**	**Adjusted R-square**	**F change P value**
	Beta	**t**	**P value**				
RNU6-1/ miR-16-5p	0.324	3.36	0.002	0.65	0.42	0.41	<0.001
miR-25-3p/ miR-320a	0.452	5.37	<0.001	0.74	0.55	0.53	0.001
Let-7e-5p/miR-15b-5p	0.313	3.40	0.002	0.81	0.66	0.64	0.001
miR-30a-5p/miR-324-5p	-0.213	-2.59	0.01	0.84	0.70	0.67	0.03
miR-17-5p/miR-194-5p	-0.217	-2.34	0.02	0.86*	0.73*	0.70*	0.02*

*Predictors: constant, RNU6-1/ miR-16-5p, miR-25-3p/miR-320a, let-7e-5p/ miR-15b-5p, miR-30a-5p/ miR324-5p, miR-17-5p/ miR-194-5p (5-ratio panel)

Table 2: Fold difference for the 5 miRNA ratios for esophageal adenocarcinoma vs. Controls (Healthy controls and individuals with Barrett's esophagus).

miRNA ratios	Cancer vs Controls		Cancer vs Barrett's	
	Fold difference	**P value**	**Fold difference**	**P value**
RNU6-1/ miR-16-5p	3.7	<0.001	3.8	<0.001
miR-25-3p/ miR-320a	1.4	0.03	2.3	0.002
Let-7e-5p/miR-15b-5p	1.6	0.002	1.8	<0.001
miR-30a-5p/miR-324-5p	0.3	<0.001	0.6	0.03
miR-17-5p/miR-194-5p	0.5	0.003	0.6	0.003

Table 3: Prediction accuracy for Cancer following stepwise inclusion of each of the 5 miRNA ratios derived from the regression model.

Model	Prediction of cancer in top 18 possibilities	AUROC (95% CI)
U6/miR-16	14	0.89 (0.79-1)
U6/miR-16 miR-25/miR-320	16	0.93 (0.83-1)
U6/miR-16 miR-25/miR-320 let-7e/ miR15b	15	0.97 (0.94-1)
U6/miR-16 miR-25/miR-320 let-7e/ miR15b miR-30a-5p/ miR-324	17	0.98 (0.96-1)
U6/miR-16 miR-25/miR-320 let-7e/ miR15b miR-30a-5p/ miR-324 miR-17/miR-194	17	0.99 (0.96-1)

Supplementary Table 1. Predicted probability that each left-out patient in the leave-one-out cross validation has cancer. Each probability was determined using a regression model of a 5 miRNA-ratio panel derived with the sequential testing method utilised in this study. Out of the 18 actual cancer patients in the cohort, The leave-one-out cross validation correctly identified 13 cancer patients from the 18 individuals (highlighted in grey) with the highest predicted probabilities of being a cancer based on the regression model.

Actual disease state	Predicted probability to be Cancer
Cancer	1.00
Cancer	1.00
Cancer	1.00
Cancer	1.00
Cancer	0.98
Control	0.97
Cancer	0.95
Barrett's	0.95
Cancer	0.90
Cancer	0.83
Control	0.80
Cancer	0.80
Control	0.80
Cancer	0.68
Cancer	0.62
Control	0.54
Cancer	0.49
Cancer	0.45
Control	0.44
Cancer	0.42
Control	0.38
Control	0.20
Barrett's	0.18
Control	0.15
Control	0.15
Cancer	0.14
Control	0.13
Control	0.12
Barrett's	0.11
Barrett's	0.09
Control	0.08
Control	0.08
Barrett's	0.08
Control	0.07
Barrett's	0.07
Control	0.05
Barrett's	0.04
Cancer	0.04
Barrett's	0.03

Cancer	0.03
Control	0.03
Control	0.02
Barrett's	0.02
Control	0.02
Control	0.02
Barrett's	0.01
Cancer	0.00

FIGURE LEGENDS

Figure 1: Study design and approach to biomarker selection.

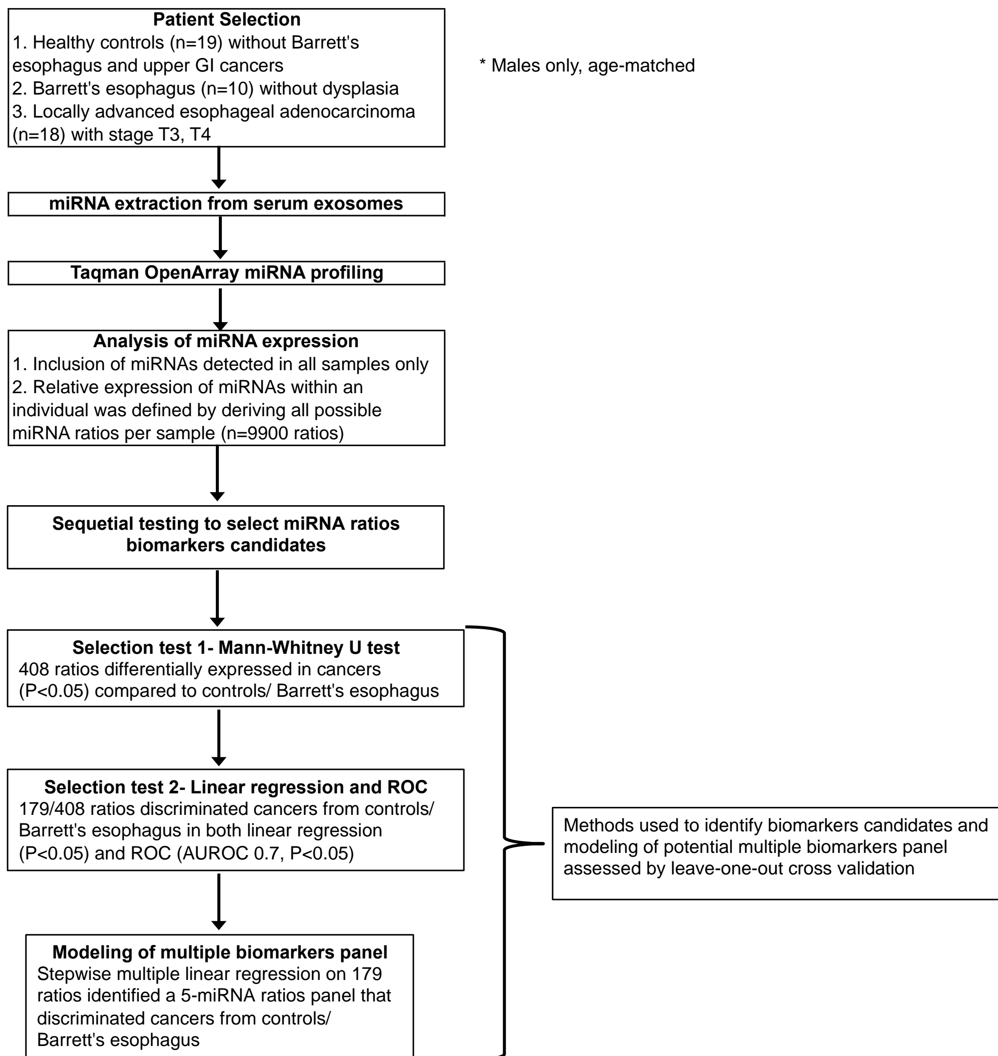
Figure 2: The expressions of 179 miRNA ratios in serum circulating exosomes were significantly different between healthy controls or patients with non-dysplastic Barrett's esophagus compared to patients with esophageal adenocarcinoma (Mann-Whitney U-test and linear regression, P<0.05). All ratios achieved an AUROC >0.7 in ROC curves for the discrimination of esophageal adenocarcinoma from controls and Barrett's esophagus.

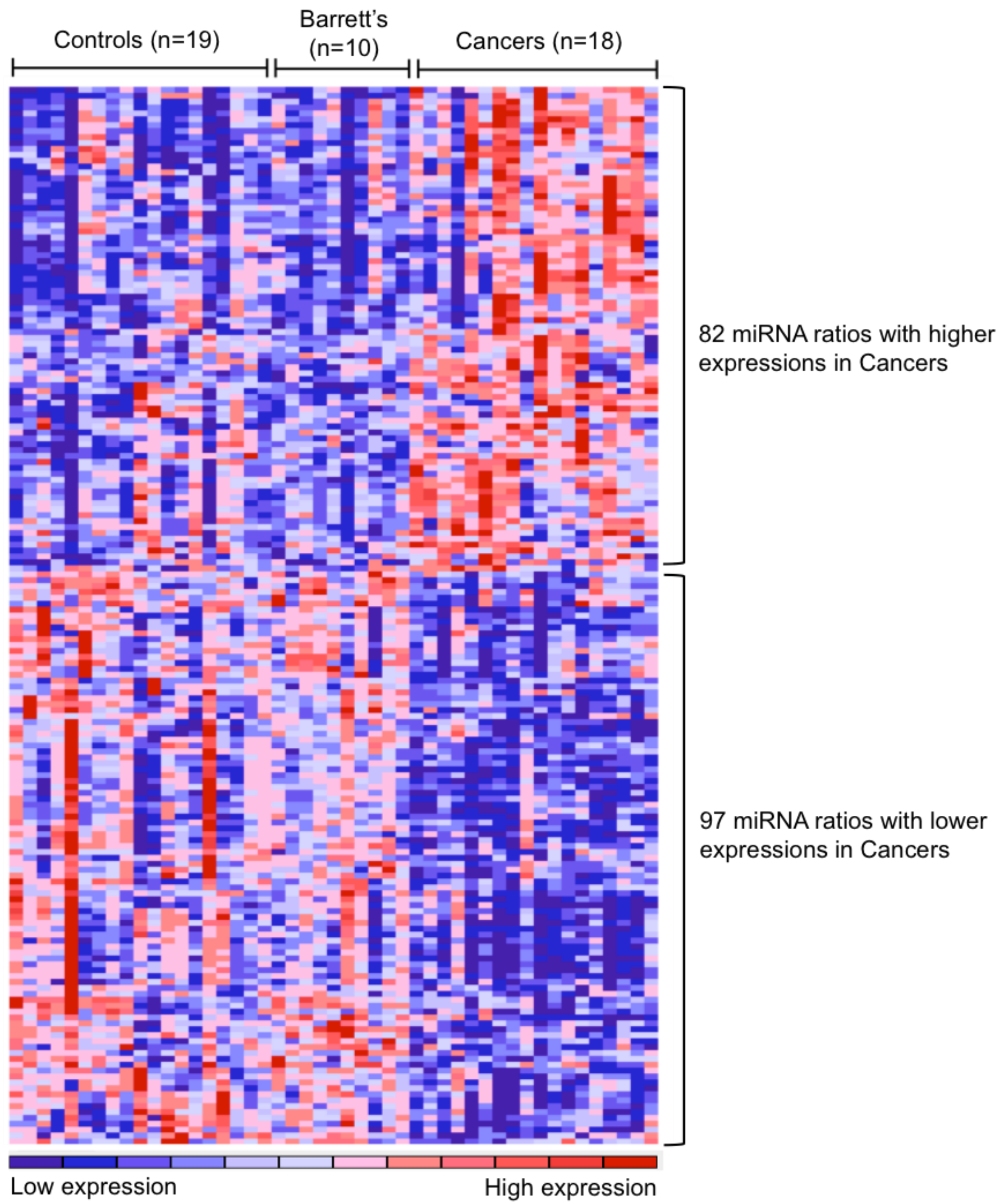
Figure 3: ROC curves for the combined 5 miRNA ratios biomarker panel compared to individual miRNA ratios as single biomarkers, demonstrating better sensitivity and specificity for identifying individuals with esophageal adenocarcinoma vs. controls.

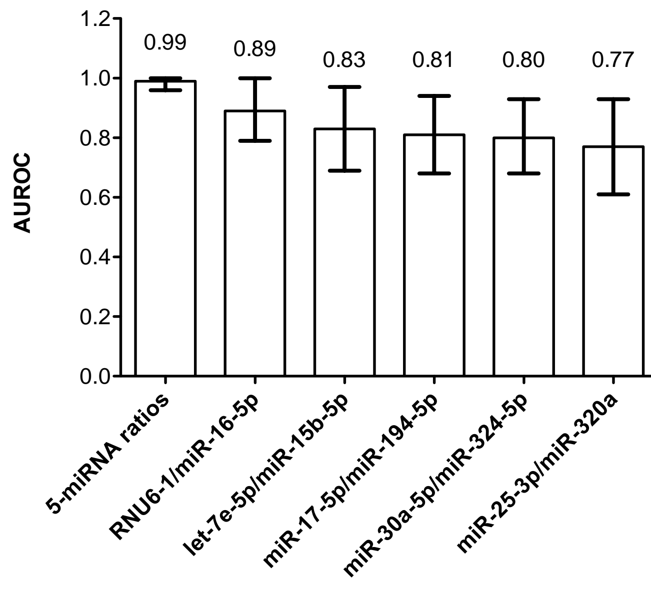
Supplementary Figure 1: Distribution (A) and normality plots (B) of the standardized residuals of the combined 5-miRNA ratios biomarker panel.

Supplementary Figure 2: Distribution of the 5 miRNA ratios in healthy controls, Barrett's esophagus and esophageal adenocarcinoma.

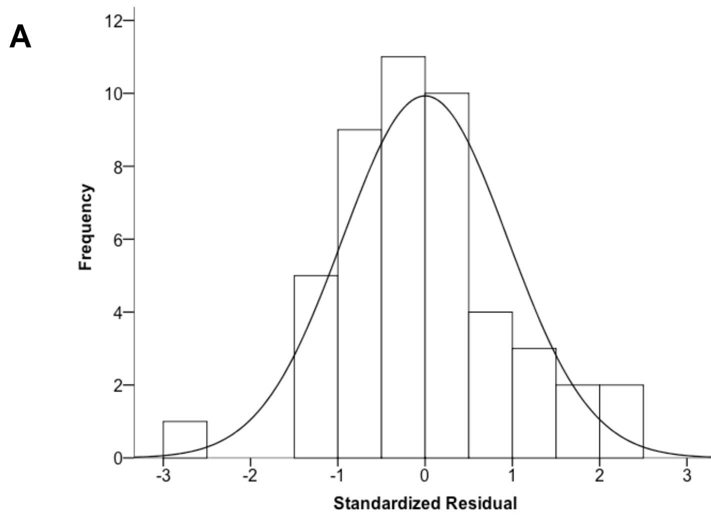
Supplementary Figure 3: LOOCV prediction errors for increasing numbers of ratios in the final regression model. Error rate is the number of cancers that were misclassified, as a percentage.


Patient Selection
1. Healthy controls (n=19) without Barrett's esophagus and upper GI cancers
2. Barrett's esophagus (n=10) without dysplasia
3. Locally advanced esophageal adenocarcinoma (n=18) with stage T3, T4
* Males only, age-matched
miRNA extraction from serum exosomes
Taqman OpenArray miRNA profiling
Analysis of miRNA expression
1. Inclusion of miRNAs detected in all samples only
2. Relative expression of miRNAs within an individual was defined by deriving all possible miRNA ratios per sample (n=9900 ratios)
Sequetial testing to select miRNA ratios biomarkers candidates
Selection test 1- Mann-Whitney U test
408 ratios differentially expressed in cancers (P<0.05) compared to controls/ Barrett's esophagus
Selection test 2- Linear regression and ROC
179/408 ratios discriminated cancers from controls/ Barrett's esophagus in both linear regression (P<0.05) and ROC (AUROC 0.7, P<0.05)
Modeling of multiple biomarkers panel
Stepwise multiple linear regression on 179 ratios identified a 5-miRNA ratios panel that discriminated cancers from controls/ Barrett's esophagus
Methods used to identify biomarkers candidates and modeling of potential multiple biomarkers panel assessed by leave-one-out cross validation

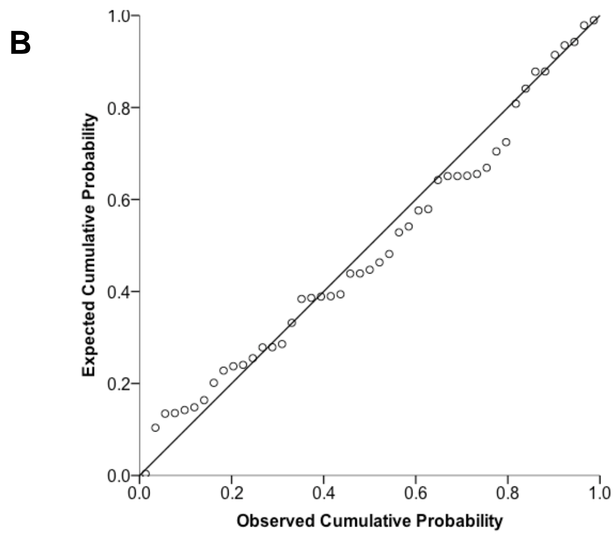

Controls (n=19)
Barrett's (n=10)
Cancers (n=18)
82 miRNA ratios with higher expressions in Cancers
97 miRNA ratios with lower expressions in Cancers
Low expression
High expression

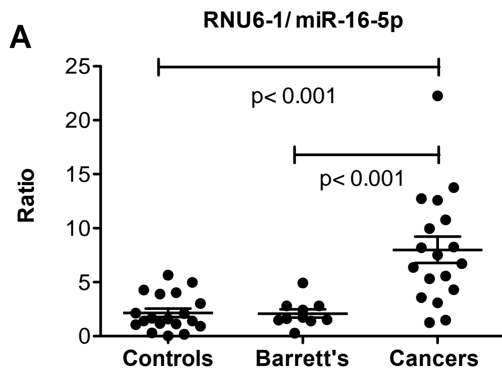

AUROC
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.99
0.89
0.83
0.81
0.80
0.77
5-miRNA ratios
RNU6-1/miR-16-5p
let-7e-5p/miR-15b-5p
miR-17-5p/miR-194-5p
miR-30a-5p/miR-324-5p
miR-25-3p/miR-320a

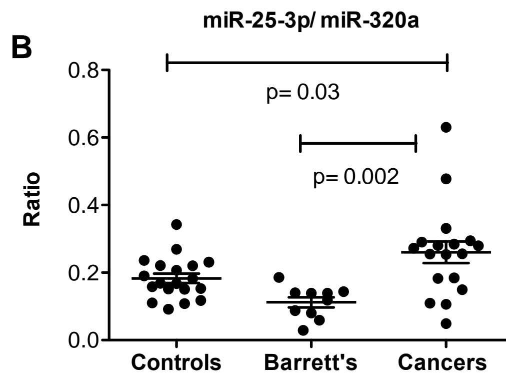
A

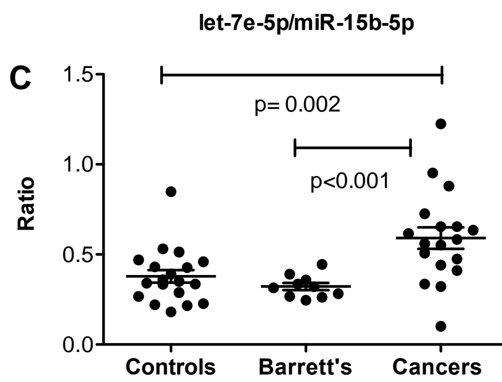


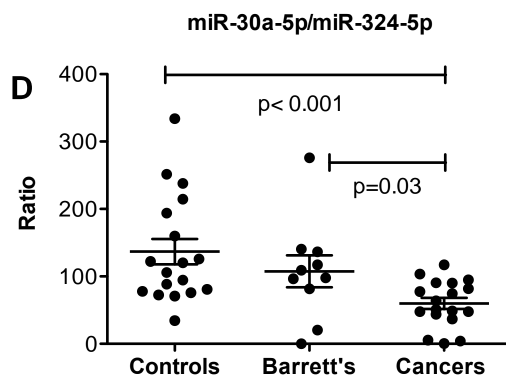
B

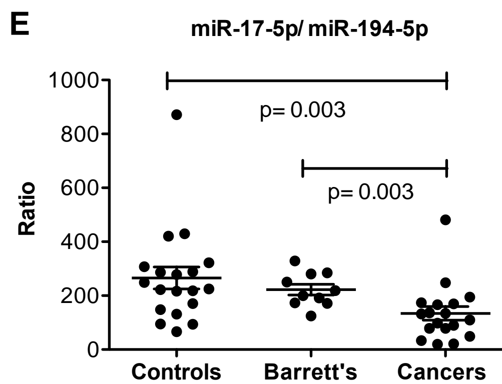


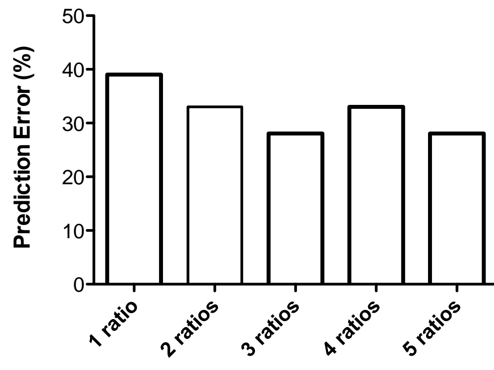

A
RNU6-1/ miR-16-5p
p< 0.001
p< 0.001
Ratio
25
20
15
10
5
0
Controls
Barrett's
Cancers


B
miR-25-3p/ miR-320a
p= 0.03
p= 0.002
Ratio
0.8
0.6
0.4
0.2
0.0
Controls
Barrett's
Cancers


C
let-7e-5p/miR-15b-5p
p= 0.002
p<0.001
Ratio
1.5
1.0
0.5
0.0
Controls
Barrett's
Cancers


D
miR-30a-5p/miR-324-5p
p< 0.001
p=0.03
Ratio
400
300
200
100
0
Controls
Barrett's
Cancers


E
miR-17-5p/ miR-194-5p
p= 0.003
p= 0.003
Ratio
1000
800
600
400
200
0
Controls
Barrett's
Cancers


Prediction Error (%)
50
40
30
20
10
0
1 ratio
2 ratios
3 ratios
4 ratios
5 ratios