Am J Cancer Res 2016;6(11):2713-2721
www.ajcr.us /ISSN:2156-6976/ajcr0037215

*Original Article*

# Circulating miRNA profile in esophageal adenocarcinoma

Keqiang Zhang[1], Xiwei Wu[2], Jinhui Wang[2], Joshua Lopez[1], Weiying Zhou[3], Lu Yang[2], Shizhen Emily Wang[3], Dan J Raz[1], Jae Y Kim[1]

[1]*Division of Thoracic Surgery,* [2]*The Integrative Genomics Core Lab of Department of Molecular Medicine,* [3]*Department of Cancer Biology, City of Hope National Medical Center, Duarte, California 91010, USA*

Received July 21, 2016; Accepted August 6, 2016; Epub November 1, 2016; Published November 15, 2016

**Abstract:** Esophageal cancer is one of the deadliest cancers worldwide. It typically has a poor overall prognosis because of diagnosis in advanced stages. Sensitive biomarkers for the early detection of esophageal cancer are urgently needed to reduce the high mortality of this disease. Circulating microRNAs (miRNAs) are emerging as a novel non-invasive biomarker for cancer diagnosis due to its high stability and specificity. In this study, we profiled circulating miRNAs in serum of patients with esophageal adenocarcinoma (EAC) using the Solexa sequencing analysis. The analysis demonstrated marked differences of more than 195 circulating miRNAs including 96 of upregulated and 99 of downregulated miRNA in EAC patients compared with healthy subjects. Quantitative reverse transcription PCR verified that the concentrations of circulating miR-25-3p and miR-151a-3p were significantly elevated, while the concentrations of miR-100-5p and miR-375 were significantly decreased in EAC patients compared with healthy controls. These data indicate the profile of these 4 miRNAs may potentially serve as a serum biomarker to identify patients with EAC, and circulating miRNA profiling may be clinically useful for early detection or treatment response in EAC patients.

**Keywords:** Esophageal adenocarcinoma (EAC), circulating microRNA (miRNA)

## Introduction

Esophageal cancer is the sixth most common cancer and the fifth leading cause of cancer death in men worldwide [1]. The two main types of esophageal cancer are squamous cell carcinoma (ESCC) or adenocarcinoma (EAC). Over the past 30 years, the frequency of EAC cases has been increasing faster than any other cancers in the United States and currently accounts for over 80% of new esophageal cancer cases in the United States [2]. Unfortunately, the majority of EAC patients are usually diagnosed at advanced stages when current therapies are largely ineffective, resulting in a very poor overall prognosis. This dismal prognosis has led to increased interest in screening strategies to identify EAC at an earlier stage. However, the only available screening options involve an endoscopic approach. This approach is invasive, prone to sampling error and subject to inter-observer and intra-observer variability [3]. Current screening approaches are not practical for the general population, thus a blood based biomarker of EAC would be a promising alternative. Such a biomarker may also meet the clinical need for a better prediction of treatment response.

MicroRNAs (miRNAs) are non-coding, small RNA molecules of 21-24 nucleotides that help regulate protein expression by forming complementary base pairs within the 3' untranslated regions of protein-encoding mRNAs, resulting in mRNA degradation or translational inhibition [4]. Abundant evidence has demonstrated that miRNAs play important roles in normal development, differentiation, growth and death [4, 5]. Because of altered epigenetic mechanisms and miRNA processing machinery, miRNAs are differentially expressed in malignant cells compared with normal cells. Altered miRNAs are proven to be involved in the initiation and progression of human cancers, and are associated

with diagnosis, staging, progression, prognosis and response to treatment [6].

It has been recently discovered that extracellular miRNAs circulate in the bloodstream; and that these circulating miRNAs are remarkably stable, since these miRNAs in serum are either enveloped by microvesicles or protected by binding proteins [7-9], and they can also be chemically modified (e.g., methylation), making them highly resistant to degradation by ribonuclease [9-11]. The origin and precise cellular releasing mechanisms of circulating miRNAs remain largely unknown. Studies have indicated that miRNAs can be released into circulation by apoptotic and necrotic cell death [10, 12]. MiRNAs can also be actively secreted by cancer cells to promote tumor growth and spread [10, 13, 14], and various cell types such as blood cells, immune effector cells, other cells from affected organs involved in tumor invasion and inflammatory response [10, 15, 16]. For example, it has been reported that cells surrounding the primary tumor can secrete tumor-suppressive miRNAs, which block tumor growth and invasion [17, 18]. These results suggest that tumor-associated miRNA profile including circulating miRNA reflect both tumor progression and systemic response of the host. Compared with healthy controls, circulating miRNA profile exhibits a distinctive difference in patients with various cancers, including lung, colorectal, prostate, and pancreatic cancers, and it potentially represents a diagnostic and prognostic biomarker for human cancers [10, 11, 19, 20]. Recently, Zhang *et al.* has showed that miRNA profile in ESCC efficiently distinguished ESCC patients from healthy controls [22]. Accumulating data have highlighted the potential significance of circulating miRNA profile as a reliable noninvasive biomarker for the clinical cancer diagnosis and prognosis.

For circulating miRNA profiles in esophageal cancer patients, the majority of studies have been focused on ESCC and very few studies about miRNAs profiles in EAC have been reported [22, 23]. The identification of specific circulating miRNAs in EAC will have important potential for early diagnosis, prognosis, and assessment of cancer therapy. Fast advances in discovery approaches, including deep sequencing analysis, have allowed for the recent identification of circulating miRNAs including these miRNAs that are only enriched in circulation but undetectable in cancer tissues. In the study, we profiled circulating miRNA signatures in EAC patients using a *de novo* deep sequencing strategy as we described previously [21]. We have demonstrated that EAC patients have a distinct circulating miRNA profile that distinguishes them from healthy controls, indicating circulating miRNA profile can serve as a noninvasive, accurate biomarker for EAC diagnosis.

## Material and method

### *Serum samples and RNA purification from serum*

After giving informed consent under an Institutional Review Board (IRB)-approved protocol from 2012 to 2013, peripheral blood was collected from 10 patients with a histologic diagnosis of stage I-III esophageal adenocarcinoma at the City of Hope Cancer Center. The serum was aliquoted and stored at -80°C until use. Serum from 11 healthy controls was purchased from BioServe (Beltsville, MD). TRIZOL LS reagent (Invitrogen) was used to extract total RNA from ~1.5 ml of serum, as described in the manufacturer's protocol. RNA pellet was dissolved in RNase-free water, and subjected to further processing.

### *Solexa deep sequencing for small RNAs*

Each serum sample was independently subjected to library preparation and deep sequencing according to the method we previously described [21]. Briefly, 5 μl of total RNA extracted from serum was used for small RNA library preparation according to the 5' ligation-dependent (5' monophosphate-dependent) manufacturer's protocol (Digital Gene Expression for small RNA; Illumina). The library was quantified using picoGreen and quantitative PCR assays. Sequencing was performed on a Genome Analyzer IIx (Illumina), and image processing and base calling were conducted using Ill-umina's pipeline. Sequenced reads from Solexa were first mapped onto human genome version hg18 using Novoalign software and the expression level of mature miRNAs in the miRBase human miRNA database V15 was summarized as described previously [24]. Normalization and identification of differentially expressed miRNAs between two groups were carried out using Bioconductor package "edgeR" [25].

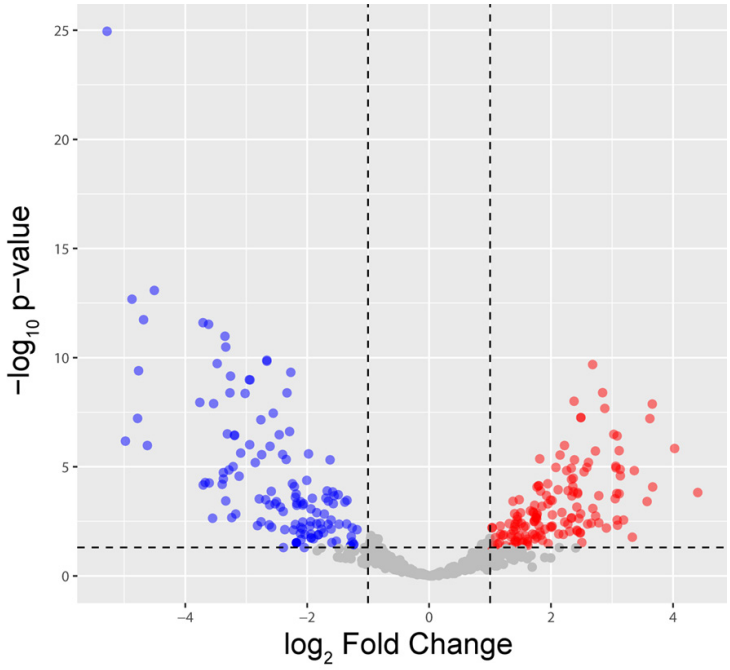



**Figure 1.** Volcano plot illustrates the frequency distribution of fold changes ($log_2$, x axis) and the distribution of fold changes with transformed *P*-values (-$log_{10}$ of *P* value, y axis). The vertical dash lines represent the cut-off levels of $log_2$ FCs <-1 or >1, respectively. The horizontal dash line shows where $P \geq 0.05$ with points above the line having $P<0.05$ and points below the line having $P>0.05$. These miRNAs having *P*-values <0.05 and with $log_2$ FCs either <-1 or >1 are shown in blue or red.

*Quantitative reverse transcription PCR (RT-qPCR)*

Levels of selected miRNAs were validated using RT-qPCR. The selected miRNAs had the largest differential expressions or were previously reported as being associated with cancer. Level of miR-16 was used as a reference for normalization, based on relative abundance, lowest coefficient of variation among all samples, and previous reports of its use as a reference for serum miRNA [26, 27]. For RT-qPCR assay, the total RNA extracted from ~1.5 ml of the same serum for deep sequencing was reversely transcribed using the miScript Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. For quantitative PCR amplification, the reaction mixture of 12.5 µl consisted of 1× SYBR Green PCR Master Mix, 1× Universal Primer, 0.25 µl cDNA and 0.2 µM of miRNA specific primer. The following primers were used: miR-16, 5'-CTAGCAGCACGTAAATATTGGCG-3', miR-151-3p, 5'-GCTAGACTGAAGCTCCTTGAGG-3'; miR-375, 5'-TTTGTTCGTTCGGCTCGCGT-3', miR-25-3p, 5'-CATTGCACTTGTCTCGGTCTGA-3', miR-100-5p, 5'-ACCCGTAGATCCGAACTTGTG-3'. The PCR protocol was: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, 70°C 1 min. Relative gene-expression quantification method was used to calculate the fold change of mRNA expression according to the comparative Ct method using miR-16 as an endogenous control. Final results were determined as follows: $2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$, where ΔCt values of the control and sample were determined by subtracting the Ct value of the target gene from the value of the reference miR-16. The level of each miRNA in each serum sample was first normalized to the average of miRNA in healthy controls; data was then presented as fold or ratio to the average.

*Statistical analysis*

After mapping the deep sequencing data onto the human genome and counting the reads for the mature miRNAs in the miRBase database, raw miRNA expression data were quantitatively normalized and $log_2$ transformed with offset of 1. Absolute $log_2$ fold change ($log_2$ FC) >1 between tumor and normal samples with a *P* value and false discovery rate (FDR) <0.05 were considered statistically significant. Group comparisons for continuous data were done with student's t-test for independent means or two-way ANOVA. The multivariate statistic Hotelling's T-square 2 test was used to compare differential miRNA profiles between healthy controls and EAC patients. Statistical significance was set at $P<0.05$.

## Results

*Identification of differential circulating miRNA profile between EAC patients and healthy controls by Selex sequencing analysis*

To develop novel non-invasive biomarkers for EAC, we first profiled miRNA profiles in serum samples from 10 EAC patients and 11 healthy controls by Solexa sequencing. Sequencing

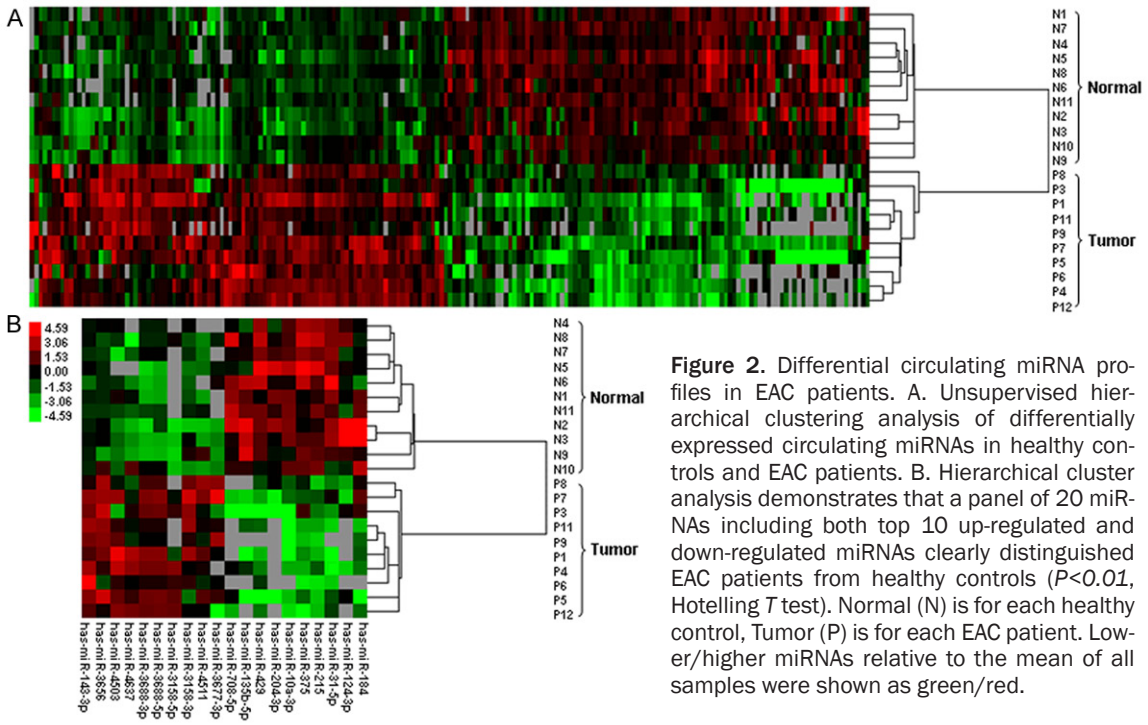



**Figure 2.** Differential circulating miRNA profiles in EAC patients. A. Unsupervised hierarchical clustering analysis of differentially expressed circulating miRNAs in healthy controls and EAC patients. B. Hierarchical cluster analysis demonstrates that a panel of 20 miRNAs including both top 10 up-regulated and down-regulated miRNAs clearly distinguished EAC patients from healthy controls (*P<0.01*, Hotelling *T* test). Normal (N) is for each healthy control, Tumor (P) is for each EAC patient. Lower/higher miRNAs relative to the mean of all samples were shown as green/red.

analysis showed that miRNAs were one of the major components of small RNAs in serum. On average, miRNAs comprised about 18% of all aligned read counts. A miRNA was considered as a real candidate, if its counts per million reads (CPM) were greater than 5 in at least 2 patients. After data filtering, a total of 686 miRNAs were efficiently detected in serum from both the EAC patients and healthy controls. Volcano plot analysis was applied to illustrate the frequency distribution of miRNA fold changes and the distribution of fold changes with corresponding *P*-values. As shown in **Figure 1**, miRNAs with fold changes either <-1 or >1 and having *P*-values <0.05 are shown in blue or red, these miRNAs represent candidates that display both large magnitude fold change and high statistical significance. In comparison to healthy controls, a total of 195 miRNAs were identified with differential expression in EAC patients with an absolute $\log_2$ FC >1.5 and FDR <0.05. Among these miRNAs, 99 had lower expression and 96 had higher expression in EAC patients compared with healthy controls (Supplementary Table 1). We used an unsupervised clustering method that was unbiased to the clinical annotations to investigate the different concentration patterns for the aforementioned 195 miRNAs in these serum samples of EAC patients and healthy controls. As shown in **Figure 2A**, unsupervised hierarchical clustering of these differentially expressed miRNAs clearly separated EAC patients from healthy controls. We further analyzed the effectiveness of these aberrant miRNAs whose fold changes were over the upper 95th percentile or below the lower 5th percentile in differentiating EAC patients from healthy controls. Hierarchical clustering analysis showed that these top upregulated and downregulated miRNAs (Supplementary Table 1) could also clearly differentiate EAC patients from healthy controls (**Figure 2B**), further indicating clinical significance of circulating miRNA profile in EAC diagnosis. Hotelling T test also demonstrated that this signature of 20 miRNAs were significantly different between these EAC samples and health control samples (*P*<0.01).

*Validation of miRNA levels by q-RT-PCR analysis*

We selected four miRNAs, including miR151a-3p, miR-375, miR25-3p, and miR100-5p for RT-qPCR-based validation using the same total RNA extracts for deep sequencing. These 4 miRNAs were selected based on large differential expressions or previous reports of associa-

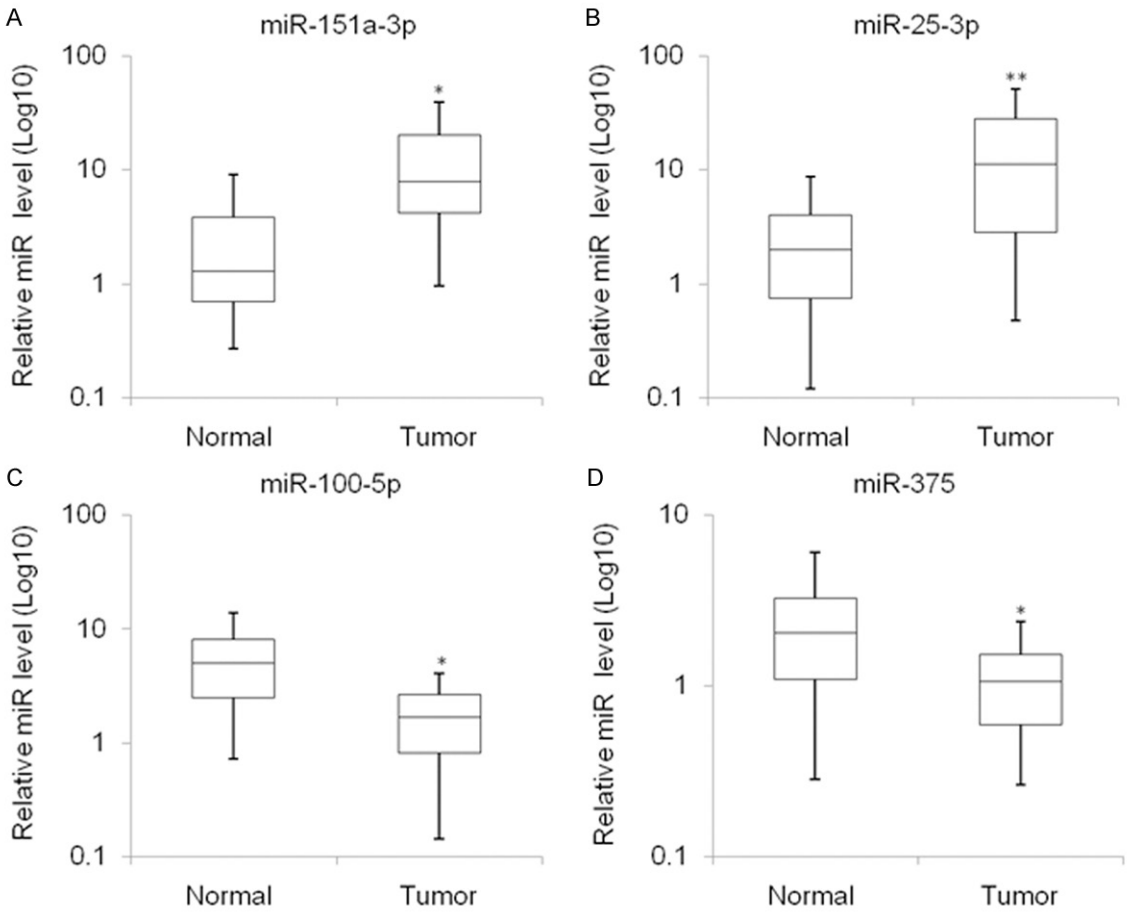



**Figure 3.** Validation of miRNAs levels by RT-qPCR analysis. The concentrations of miRNAs in serum were measured by RT-qPCR, and the miRNAs were calculated as $2^{-\Delta Ct}$ to miR-16. Box and Whisker plots of RT-qPCR results for A. miR-151a-3p, B. miR-25, C. miR-100-5p, and D. miR-375, the results are presented in the $\log_{10}$ scale. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75$^{th}$, 25$^{th}$ percentiles, and the median, respectively. Student *t*-test was performed to analyze the difference between circulating miRNAs in EAC patients and healthy controls ("*" for $P<0.05$ and "**" for $P<0.01$ compared to healthy controls).

tion with cancer. For PCR analyses, miR-16, which was consistent among all samples, and reportedly used as the internal control for circulating miRNAs in previous PCR-based studies [26, 27], was used as the reference for data normalization. The RT-qPCR assay for measuring serum miRNA concentration was validated using miRNAs extracted from cell lines and proved reliable and reproducible. Pairwise Pearson correlation was calculated to determine the consistency of the miRNA levels determined by deep sequencing (normalized $\log_2$ counts and PCR-determined levels relative to miR-16) in each sample. For all tested miRNAs, significant correlations ($P<0.05$) were observed between the two methods. Consistent with results of Solexa sequencing, RT-qPCR analysis identified the concentrations of miR-151a-3p (**Figure 3A**) and miR-25-3p (**Figure 3B**) were significantly higher ($P<0.05$), while miR-100-5p (**Figure 3C**) and miR-375 (**Figure 3D**) were significantly lower ($P<0.05$) in EAC patients compared to healthy controls. Hierarchical clustering analysis revealed that these 4 miRNAs also efficiently differentiated these EAC patients from healthy controls (Supplementary Figure 1, $P<0.05$). Therefore, this data indicated the profile of these 4 miRNAs may potentially serve as serum biomarker for EAC diagnosis.

## Discussion

In this study, we have for the first time demonstrated marked differences of more than 195 circulating miRNAs including 96 of upregulated and 99 of downregulated miRNA in EAC pa-

tients compared with healthy controls. Using RT-qPCR, we have confirmed that the concentrations of circulating miR-25, miR-151, miR-100, and miR-375 were significantly different in EAC patients compared to healthy controls. Because of the common biology among various tumors including unlimited proliferation and rapid metastasis, many of these differential circulating miRNAs in EAC including miR-25, miR-151, miR-100, miR-375 are likely to be observed in the serum of patients with other types of tumors including ESCC [22]. Similar to oncogenes, a partial of miRNAs play oncogeneitc roles in tumorigenesis. For example, several studies demonstrated that miR-25, miR-93, and miR-106b encoded by the miR-106b-25 polycistron exhibit potential proliferative, anti-apoptotic, cell cycle-promoting effects *in vitro* and tumorigenic activity *in vivo* via targeting p21 and Bcl-2 interacting mediator of cell death (Bim) [28, 29]. Xu *et al* recently demonstrated that miR-25 directly targets CDH1 tumor suppressor in esophageal cancer to promote invasion [29]. Contrast to miR-25, miR-375 functions as a tumor suppressor; it inhibits clonogenicity, cell motility, cell proliferation, tumor formation and metastasis of esophageal cancer in mice. MiR-375 also targets 3-phosphoinositide-dependent protein kinase-1 (PDK1) in esophageal cancer, resulting in enhanced metabolism and proliferation [30]. Furthermore, miR-375 interacts with the 3'-untranslated region of IGF1R and down-regulate its expression *in vitro*, of note, the expression of IGF1R was also negatively correlated with miR-375 expression in clinical samples [31]. Over all, these studies have revealed the important roles of dysregulated miRNAs in various human cancers progression.

Deregulation of miRNA expression was originally found in esophageal cancer tissues. Feber *et al.* first compared the expression of 328 human miRNAs in both EAC and ESCC tissues, and they found that 13 miRNAs were significantly altered in EAC tissues compared to normal tissues, such as upregulation of miR-21, miR-192, miR-194, miR-93 and downregulation of miR-203, miR-205, miR-27b, miR-100, miR-125b, let-7c [32]. Kan *et al.* showed that the miR-106b-25 members were up-regulated by gene amplification, and imiR-100, miR-125b, miR-205, and miR-19b were significantly down-regulated in EAC tissues, compared to normal squamous epithelial tissues [28]. Similarly, Guo *et al.* also reported that miR-25, miR-424, and miR-151 were upregulated and miR-100, miR-99a, and miR-29c were downregualted in ESCC versus normal tissues [33]. Ogawa *et al.* found that 21 miRNAs were upregulated at least 2-fold, including aforementioned miR-21, miR-25, and miR-151; and other miRNAs are downregulated at least 2-fold including miR-375, miR-100 in EAC [34].

Interestingly, the levels of some miRNAs differentially expressed in EAC tissues were also altered in serum of EAC patients compared with healthy controls. For example, an earlier study by Tanaka *et al.* showed that the levels of circulating miR-21, miR-145, miR-200c, and let-7c were significantly higher in esophageal cancer patients than healthy controls [35]. Similarly, another study reported that level of miR-107 was significantly decreased in ESCC patients, while downregulation of miR-107 was observed in neoplastic and preneoplastic esophageal tissues [36]. In this study, we verified that circulating miR-25 and miR-151 were significantly increased, while miR-100 and miR-375 were significantly decreased in EAC patients. Komatsu *et al.* reported the upregulation of miR-21 and downregulation of miR-375 in serums of ESCC patients compared to healthy controls [37]. Moreover, the same group further demonstrated that high level of circulating miR-21 was correlated with higher risk of recurrence and poorer survival, whereas high level of circulating miR-375 was indicative of better survival [37, 38]. In agreement with above findings, Kong *et al.* also revealed an inverse correlation of levels of circulating miR-375 with poor survival in ESCC [31]. Similarly, a study by Nguyen *et al*. also indicated that miR-375 was a prognostic factor for EAC patients EAC [39]. We previously also demonstrated that levels of circulating miR-375 with miR-122 could serve as prognostic biomarkers for neoadjuvant chemotherapy response in breast cancer [21]. In general, the exact mechanisms for these changes in circulating miRNA profile remain largely unknown. Both alterations in tumor cells and complex bidirectional interactions between tumor cells and host cells may be responsible for the altered circulating miRNA profile in tumor patients. For example, frequent

promoter hypermethylation of miR-375 was found in both ESCC and EAC tissues [30, 31].

In this pilot study, we demonstrated that EAC patients have a distinctive circulating miRNA profile compared with healthy controls. This circulating miRNA profile especially a profile with a limited number of miRNAs has the potential to be a biomarker for the diagnosis and detection of EAC, and warrants further investigation and validation with a large number of EAC samples. Future studies are also needed to identify the physiological functions of circulating miRNAs and their relationship with tumorigenesis of EAC.

## Acknowledgements

The authors acknowledge Youqiang Li for total RNA extraction from serum. Research reported in this publication is supported by the National Cancer Institute of the National Institutes of Health under award numbers NIH 5K12-CA001727-20 (DR), P30CA33572 through the use of the City of Hope Integrative Genomics Core, and the generous support of the Baum Family Foundation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Jae Y Kim, Department of Surgery, Division of Thoracic Surgery, City of Hope Cancer Center, 1500 East Duarte Rd, Duarte, CA 91010, USA. Tel: 626-471-7287; Fax: 626-471-6285; E-mail: jaekim@coh.org

## References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.

[2] Pohl H, Welch HG. The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence. J Natl Cancer Inst 2005; 97: 142-6.

[3] Shah AK, Saunders NA, Barbour AP, Hill MM. Early diagnostic biomarkers for esophageal adenocarcinoma--the current state of play. Cancer Epidemiol Biomarkers Prev 2013; 22: 1185-209.

[4] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-97.

[5] Heinrich EM, Dimmeler S. MicroRNAs and stem cells. control of pluripotency, reprogramming, and lineage commitment. Circ Res 2012; 110: 1014-22.

[6] Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857-66.

[7] Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, de Gruijl TD, Wurdinger T, Middeldorp JM. Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci U S A 2010; 107: 6328-33.

[8] Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007; 9: 654-9.

[9] Chim SS, Shing TK, Hung EC, Leung TY, Lau TK, Chiu RW, Lo YM. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem 2008; 54: 482-90.

[10] Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Zhang Y, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Wang J, Zen K, Zhang J, Zhang CY. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 2008; 18: 997-1006.

[11] Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008; 105: 10513-8.

[12] Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin GA. MicroRNAs in body fluids--the mix of hormones and biomarkers. Nat Rev Clin Oncol 2011; 8: 467-77.

[13] Zhou W, Fong MY, Min Y, Somlo G, Liu L, Palomares MR, Yu Y, Chow A, O'Connor ST, Chin AR, Yen Y, Wang Y, Marcusson EG, Chu P, Wu J, Wu X, Li AX, Li Z, Gao H, Ren X, Boldin MP, Lin PC, Wang SE. Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. Cancer Cell 2014; 25: 501-15.

[14] Fabbri M, Paone A, Calore F, Galli R, Gaudio E, Santhanam R, Lovat F, Fadda P, Mao C, Nuovo GJ, Zanesi N, Crawford M, Ozer GH, Wernicke D, Alder H, Caligiuri MA, Nana-Sinkam P, Perrotti D, Croce CM. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. Proc Natl Acad Sci U S A 2012; 109: E2110-6.

[15] Okada H, Kohanbash G, Lotze MT. MicroRNAs in immune regulation--opportunities for cancer immunotherapy. Int J Biochem Cell Biol 2010; 42: 1256-61.
[16] Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. Clin Chim Acta 2001; 313: 139-42.
[17] Ha TY. The Role of MicroRNAs in Regulatory T Cells and in the Immune Response. Immune Netw 2011; 11: 11-41.
[18] Toffanin S, Sia D, Villanueva A. microRNAs: new ways to block tumor angiogenesis? J Hepatol 2012; 57: 490-1.
[19] Schwarzenbach H, Nishida N, Calin GA, Pantel K. Clinical relevance of circulating cell-free microRNAs in cancer. Nat Rev Clin Oncol 2014; 11: 145-56.
[20] Fiorucci G, Chiantore MV, Mangino G, Percario ZA, Affabris E, Romeo G. Cancer regulator microRNA: potential relevance in diagnosis, prognosis and treatment of cancer. Curr Med Chem 2012; 19: 461-74.
[21] Zhang C, Wang C, Chen X, Yang C, Li K, Wang J, Dai J, Hu Z, Zhou X, Chen L, Zhang Y, Li Y, Qiu H, Xing J, Liang Z, Ren B, Yang C, Zen K, Zhang CY. Expression profile of microRNAs in serum: a fingerprint for esophageal squamous cell carcinoma. Clin Chem 2010; 56: 1871-9.
[22] Gu J, Wang Y, Wu X. MicroRNA in the pathogenesis and prognosis of esophageal cancer. Curr Pharm Des 2013; 19: 1292-300.
[23] Wu X, Somlo G, Yu Y, Palomares MR, Li AX, Zhou W, Chow A, Yen Y, Rossi JJ, Gao H, Wang J, Yuan YC, Frankel P, Li S, Ashing-Giwa KT, Sun G, Wang Y, Smith R, Robinson K, Ren X, Wang SE. De novo sequencing of circulating miRNAs identifies novel markers predicting clinical outcome of locally advanced breast cancer. J Transl Med 2012; 10: 42.
[24] Weng L, Wu X, Gao H, Mu B, Li X, Wang JH, Guo C, Jin JM, Chen Z, Covarrubias M, Yuan YC, Weiss LM, Wu H. MicroRNA profiling of clear cell renal cell carcinoma by whole-genome small RNA deep sequencing of paired frozen and formalin-fixed, paraffin-embedded tissue specimens. J Pathol 2010; 222: 41-51.
[25] Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010; 26: 139-40.
[26] Pescador N, Perez-Barba M, Ibarra JM, Corbaton A, Martinez-Larrad MT, Serrano-Rios M. Serum circulating microRNA profiling for identification of potential type 2 diabetes and obesity biomarkers. PLoS One 2013; 8: e77251.
[27] Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, Wrang Teilum M, Dahlsveen IK. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. Methods 2013; 59: S1-6.
[28] Kan T, Sato F, Ito T, Matsumura N, David S, Cheng Y, Agarwal R, Paun BC, Jin Z, Olaru AV, Selaru FM, Hamilton JP, Yang J, Abraham JM, Mori Y, Meltzer SJ. The miR-106b-25 polycistron, activated by genomic amplification, functions as an oncogene by suppressing p21 and Bim. Gastroenterology 2009; 136: 1689-700.
[29] Xu X, Chen Z, Zhao X, Wang J, Ding D, Wang Z, Tan F, Tan X, Zhou F, Sun J, Sun N, Gao Y, Shao K, Li N, Qiu B, He J. MicroRNA-25 promotes cell migration and invasion in esophageal squamous cell carcinoma. Biochem Biophys Res Commun 2012; 421: 640-5.
[30] Li X, Lin R, Li J. Epigenetic silencing of microRNA-375 regulates PDK1 expression in esophageal cancer. Dig Dis Sci 2011; 56: 2849-56.
[31] Kong KL, Kwong DL, Chan TH, Law SY, Chen L, Li Y, Qin YR, Guan XY. MicroRNA-375 inhibits tumour growth and metastasis in oesophageal squamous cell carcinoma through repressing insulin-like growth factor 1 receptor. Gut 2012; 61: 33-42.
[32] Feber A, Xi L, Luketich JD, Pennathur A, Landreneau RJ, Wu M, Swanson SJ, Godfrey TE, Litle VR. MicroRNA expression profiles of esophageal cancer. J Thorac Cardiovasc Surg 2008; 135: 255-60; discussion 60.
[33] Guo Y, Chen Z, Zhang L, Zhou F, Shi S, Feng X, Li B, Meng X, Ma X, Luo M, Shao K, Li N, Qiu B, Mitchelson K, Cheng J, He J. Distinctive microRNA profiles relating to patient survival in esophageal squamous cell carcinoma. Cancer Res 2008; 68: 26-33.
[34] Ogawa R, Ishiguro H, Kuwabara Y, Kimura M, Mitsui A, Katada T, Harata K, Tanaka T, Fujii Y. Expression profiling of micro-RNAs in human esophageal squamous cell carcinoma using RT-PCR. Med Mol Morphol 2009; 42: 102-9.
[35] Tanaka K, Miyata H, Yamasaki M, Sugimura K, Takahashi T, Kurokawa Y, Nakajima K, Takiguchi S, Mori M, Doki Y. Circulating miR-200c levels significantly predict response to chemotherapy and prognosis of patients undergoing neoadjuvant chemotherapy for esophageal cancer. Ann Surg Oncol 2013; 20 Suppl 3: S607-15.
[36] Liu R, Liao J, Yang M, Shi Y, Peng Y, Wang Y, Pan E, Guo W, Pu Y, Yin L. Circulating miR-155 expression in plasma: a potential biomarker for early diagnosis of esophageal cancer in humans. J Toxicol Environ Health A 2012; 75: 1154-62.
[37] Komatsu S, Ichikawa D, Takeshita H, Tsujiura M, Morimura R, Nagata H, Kosuga T, Iitaka D, Konishi H, Shiozaki A, Fujiwara H, Okamoto K, Otsuji E. Circulating microRNAs in plasma of

patients with oesophageal squamous cell carcinoma. Br J Cancer 2011; 105: 104-11.

[38] Komatsu S, Ichikawa D, Takeshita H, Konishi H, Nagata H, Hirajima S, Kawaguchi T, Arita T, Shiozaki A, Fujiwara H, Okamoto K, Otsuji E. Prognostic impact of circulating miR-21 and miR-375 in plasma of patients with esophageal squamous cell carcinoma. Expert Opin Biol Ther 2012; 12 Suppl 1: S53-9.

[39] Nguyen GH, Schetter AJ, Chou DB, Bowman ED, Zhao R, Hawkes JE, Mathe EA, Kumamoto K, Zhao Y, Budhu A, Hagiwara N, Wang XW, Miyashita M, Casson AG, Harris CC. Inflammatory and microRNA gene expression as prognostic classifier of Barrett's-associated esophageal adenocarcinoma. Clin Cancer Res 2010; 16: 5824-34.

**Supplementary Table 1.** List of differential miRNAs in circulation of EAC patients

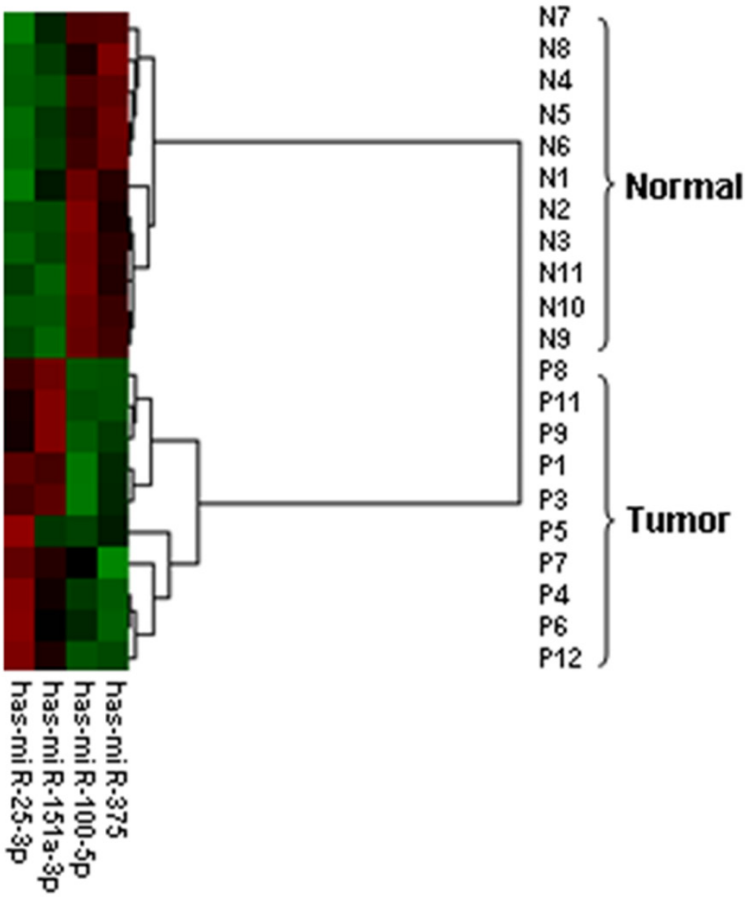
| miRNA_ID | logFC* | *P* Value | FDR |
|---|---|---|---|
| hsa-miR-184 | -6.69 | 3.8E-07 | 4.7E-06 |
| hsa-miR-124-3p | -6.30 | 5.9E-07 | 6.8E-06 |
| hsa-miR-31-5p | -5.61 | 8.6E-11 | 4.0E-09 |
| hsa-miR-10a-3p | -5.14 | 1.1E-17 | 3.8E-15 |
| hsa-miR-215 | -4.88 | 5.1E-20 | 3.5E-17 |
| hsa-miR-204-3p | -4.84 | 3.3E-06 | 2.9E-05 |
| hsa-miR-135b-5p | -4.82 | 1.0E-06 | 1.1E-05 |
| hsa-miR-375 | -4.74 | 2.6E-09 | 4.7E-08 |
| hsa-miR-708-5p | -4.69 | 1.7E-06 | 1.6E-05 |
| hsa-miR-429 | -4.57 | 3.4E-10 | 1.1E-08 |
| hsa-miR-208a | -4.45 | 1.6E-06 | 1.5E-05 |
| hsa-miR-202-3p | -4.12 | 4.4E-04 | 1.9E-03 |
| hsa-miR-516b-5p | -3.97 | 8.6E-04 | 3.1E-03 |
| hsa-miR-10b-3p | -3.92 | 1.0E-16 | 2.3E-14 |
| hsa-miR-100-5p | -3.76 | 1.3E-15 | 2.3E-13 |
| hsa-miR-200a-3p | -3.68 | 1.2E-09 | 2.5E-08 |
| hsa-miR-195-3p | -3.65 | 7.6E-04 | 2.9E-03 |
| hsa-miR-138-5p | -3.65 | 7.2E-05 | 4.4E-04 |
| hsa-miR-208b | -3.63 | 4.8E-10 | 1.4E-08 |
| hsa-miR-129-2-3p | -3.60 | 5.3E-04 | 2.2E-03 |
| hsa-miR-200b-5p | -3.49 | 1.3E-04 | 7.0E-04 |
| hsa-miR-455-3p | -3.47 | 5.6E-05 | 3.6E-04 |
| hsa-miR-141-3p | -3.42 | 1.5E-06 | 1.5E-05 |
| hsa-miR-3120-5p | -3.42 | 1.0E-07 | 1.4E-06 |
| hsa-miR-214-3p | -3.42 | 1.1E-07 | 1.4E-06 |
| hsa-miR-193b-5p | -3.40 | 6.2E-06 | 5.0E-05 |
| hsa-miR-99a-5p | -3.36 | 1.0E-14 | 1.2E-12 |
| hsa-miR-10a-5p | -3.30 | 1.5E-10 | 6.2E-09 |
| hsa-miR-195-5p | -3.28 | 6.4E-10 | 1.6E-08 |
| hsa-miR-337-5p | -3.26 | 4.1E-10 | 1.2E-08 |
| hsa-miR-150-5p | -3.26 | 3.3E-13 | 3.3E-11 |
| hsa-miR-30c-2-3p | -3.24 | 3.0E-07 | 3.7E-06 |
| hsa-miR-5683 | -3.20 | 1.8E-04 | 9.6E-04 |
| hsa-miR-592 | -3.18 | 3.8E-04 | 1.7E-03 |
| hsa-miR-125b-5p | -3.12 | 2.1E-06 | 1.9E-05 |
| hsa-miR-577 | -3.10 | 1.8E-04 | 9.4E-04 |
| hsa-miR-452-3p | -3.09 | 4.6E-06 | 3.9E-05 |
| hsa-miR-205-5p | -3.09 | 3.0E-08 | 4.5E-07 |
| hsa-miR-129-5p | -2.98 | 7.8E-06 | 6.1E-05 |
| hsa-miR-222-5p | -2.98 | 3.6E-04 | 1.6E-03 |
| hsa-miR-203 | -2.96 | 4.3E-12 | 2.3E-10 |
| hsa-miR-3545-5p | -2.95 | 3.4E-12 | 2.0E-10 |
| hsa-miR-95 | -2.94 | 1.3E-08 | 2.1E-07 |
| hsa-miR-125b-1-3p | -2.90 | 4.8E-04 | 2.0E-03 |
| hsa-miR-1228-3p | -2.87 | 2.3E-03 | 7.7E-03 |
| hsa-miR-193b-3p | -2.87 | 7.0E-07 | 7.6E-06 |
| hsa-miR-548ak | -2.85 | 8.1E-04 | 3.1E-03 |

| | | | |
|---|---|---|---|
| hsa-miR-497-5p | -2.80 | 1.2E-06 | 1.2E-05 |
| hsa-miR-452-5p | -2.74 | 2.1E-09 | 4.0E-08 |
| hsa-miR-499a-5p | -2.74 | 2.7E-10 | 9.2E-09 |
| hsa-miR-499b-3p | -2.74 | 2.5E-10 | 9.0E-09 |
| hsa-miR-204-5p | -2.73 | 7.7E-05 | 4.7E-04 |
| hsa-miR-642b-5p | -2.73 | 5.8E-04 | 2.3E-03 |
| hsa-miR-642a-3p | -2.65 | 4.7E-04 | 2.0E-03 |
| hsa-miR-511 | -2.64 | 2.8E-04 | 1.3E-03 |
| hsa-miR-206 | -2.62 | 7.0E-04 | 2.8E-03 |
| hsa-miR-125b-2-3p | -2.61 | 2.2E-04 | 1.1E-03 |
| hsa-miR-5588-5p | -2.58 | 1.3E-04 | 6.9E-04 |
| hsa-miR-9-3p | -2.57 | 3.2E-09 | 5.6E-08 |
| hsa-miR-455-5p | -2.57 | 6.5E-06 | 5.2E-05 |
| hsa-miR-99b-5p | -2.52 | 1.3E-09 | 2.6E-08 |
| hsa-miR-2116-3p | -2.50 | 2.4E-03 | 7.9E-03 |
| hsa-miR-337-3p | -2.50 | 4.4E-04 | 1.9E-03 |
| hsa-miR-320b | -2.46 | 1.6E-06 | 1.5E-05 |
| hsa-miR-30a-5p | -2.40 | 9.1E-08 | 1.3E-06 |
| hsa-miR-320e | -2.37 | 7.1E-04 | 2.8E-03 |
| hsa-miR-323a-3p | -2.36 | 2.6E-04 | 1.3E-03 |
| hsa-miR-200b-3p | -2.34 | 7.9E-04 | 3.0E-03 |
| hsa-miR-150-3p | -2.33 | 3.5E-06 | 3.0E-05 |
| hsa-miR-10b-5p | -2.32 | 4.8E-07 | 5.7E-06 |
| hsa-miR-494 | -2.31 | 4.5E-05 | 2.9E-04 |
| hsa-let-7b-3p | -2.31 | 2.5E-08 | 3.8E-07 |
| hsa-miR-508-3p | -2.28 | 3.0E-03 | 9.7E-03 |
| hsa-miR-23b-3p | -2.27 | 3.5E-12 | 2.0E-10 |
| hsa-miR-433 | -2.26 | 9.8E-04 | 3.6E-03 |
| hsa-miR-335-5p | -2.26 | 4.3E-09 | 7.2E-08 |
| hsa-miR-193a-5p | -2.23 | 2.7E-04 | 1.3E-03 |
| hsa-miR-23b-5p | -2.18 | 4.6E-04 | 1.9E-03 |
| hsa-miR-133a | -2.16 | 3.7E-04 | 1.7E-03 |
| hsa-miR-409-5p | -2.14 | 4.3E-04 | 1.9E-03 |
| hsa-miR-3591-3p | -2.10 | 1.5E-03 | 5.1E-03 |
| hsa-miR-122-5p | -2.10 | 1.5E-03 | 5.1E-03 |
| hsa-miR-369-3p | -2.07 | 2.2E-05 | 1.6E-04 |
| hsa-miR-214-5p | -2.02 | 1.3E-03 | 4.6E-03 |
| hsa-miR-135a-5p | -2.02 | 2.5E-03 | 8.3E-03 |
| hsa-miR-30a-3p | -1.98 | 2.0E-05 | 1.4E-04 |
| hsa-miR-1 | -1.96 | 2.4E-04 | 1.2E-03 |
| hsa-miR-125a-5p | -1.91 | 2.0E-03 | 6.6E-03 |
| hsa-miR-126-3p | -1.91 | 7.1E-07 | 7.7E-06 |
| hsa-miR-320c | -1.89 | 2.6E-04 | 1.3E-03 |
| hsa-miR-320d | -1.82 | 4.6E-04 | 1.9E-03 |
| hsa-miR-139-5p | -1.71 | 2.8E-04 | 1.3E-03 |
| hsa-miR-127-3p | -1.70 | 2.0E-03 | 6.7E-03 |
| hsa-miR-410 | -1.68 | 1.4E-03 | 5.0E-03 |
| hsa-miR-221-3p | -1.67 | 2.3E-06 | 2.1E-05 |
| hsa-miR-132-3p | -1.61 | 3.4E-04 | 1.5E-03 |
| hsa-miR-23a-3p | -1.61 | 1.1E-05 | 8.7E-05 |

| | | | |
|---|---|---|---|
| hsa-miR-126-5p | -1.57 | 1.2E-04 | 6.8E-04 |
| hsa-miR-145-3p | -1.51 | 1.0E-03 | 3.7E-03 |
| hsa-miR-548d-5p | 1.54 | 1.9E-04 | 9.8E-04 |
| hsa-miR-185-3p | 1.55 | 2.8E-04 | 1.3E-03 |
| hsa-miR-548e | 1.59 | 8.7E-05 | 5.2E-04 |
| hsa-miR-548ap-3p | 1.60 | 1.1E-04 | 6.3E-04 |
| hsa-miR-197-3p | 1.61 | 1.5E-04 | 8.4E-04 |
| hsa-miR-328 | 1.62 | 8.3E-04 | 3.1E-03 |
| hsa-miR-140-3p | 1.62 | 1.7E-05 | 1.3E-04 |
| hsa-miR-744-5p | 1.64 | 1.8E-04 | 9.7E-04 |
| hsa-miR-651 | 1.64 | 2.1E-03 | 7.0E-03 |
| hsa-miR-101-3p | 1.65 | 4.2E-07 | 5.1E-06 |
| hsa-miR-532-5p | 1.66 | 1.1E-05 | 8.2E-05 |
| hsa-miR-148b-3p | 1.67 | 5.8E-05 | 3.6E-04 |
| hsa-miR-148a-3p | 1.69 | 8.9E-05 | 5.3E-04 |
| hsa-miR-18b-5p | 1.71 | 1.8E-04 | 9.4E-04 |
| hsa-miR-151a-3p | 1.73 | 3.9E-05 | 2.5E-04 |
| hsa-miR-4732-5p | 1.73 | 2.0E-04 | 1.0E-03 |
| hsa-miR-106a-5p | 1.74 | 1.2E-05 | 9.3E-05 |
| hsa-miR-106b-5p | 1.74 | 6.3E-07 | 7.0E-06 |
| hsa-miR-3613-5p | 1.77 | 2.4E-04 | 1.2E-03 |
| hsa-miR-18a-5p | 1.79 | 1.7E-06 | 1.6E-05 |
| hsa-miR-33b-5p | 1.80 | 8.2E-04 | 3.1E-03 |
| hsa-miR-425-3p | 1.82 | 7.6E-06 | 6.0E-05 |
| hsa-miR-3611 | 1.87 | 6.8E-05 | 4.2E-04 |
| hsa-miR-186-5p | 1.89 | 8.7E-10 | 2.1E-08 |
| hsa-miR-223-3p | 1.89 | 1.7E-05 | 1.2E-04 |
| hsa-miR-15b-5p | 1.90 | 3.6E-06 | 3.1E-05 |
| hsa-miR-106a-3p | 1.91 | 5.5E-05 | 3.5E-04 |
| hsa-miR-450b-5p | 1.93 | 8.3E-04 | 3.1E-03 |
| hsa-miR-4746-5p | 1.93 | 5.5E-04 | 2.2E-03 |
| hsa-miR-1180 | 1.95 | 2.2E-04 | 1.1E-03 |
| hsa-miR-550a-3-5p | 1.97 | 1.8E-05 | 1.3E-04 |
| hsa-miR-550b-3p | 2.01 | 1.3E-05 | 9.7E-05 |
| hsa-miR-4286 | 2.01 | 3.6E-05 | 2.4E-04 |
| hsa-miR-1255b-5p | 2.02 | 7.6E-04 | 2.9E-03 |
| hsa-miR-185-5p | 2.06 | 1.5E-06 | 1.5E-05 |
| hsa-let-7b-5p | 2.06 | 5.0E-06 | 4.1E-05 |
| hsa-miR-550a-5p | 2.11 | 1.8E-05 | 1.3E-04 |
| hsa-miR-580 | 2.13 | 3.4E-04 | 1.5E-03 |
| hsa-miR-19b-3p | 2.15 | 1.7E-09 | 3.4E-08 |
| hsa-miR-17-5p | 2.17 | 1.2E-09 | 2.5E-08 |
| hsa-miR-93-3p | 2.18 | 2.0E-07 | 2.6E-06 |
| hsa-miR-5001-3p | 2.19 | 3.5E-04 | 1.6E-03 |
| hsa-let-7i-5p | 2.21 | 5.4E-10 | 1.4E-08 |
| hsa-miR-106b-3p | 2.22 | 1.0E-09 | 2.3E-08 |
| hsa-miR-1976 | 2.22 | 2.8E-05 | 1.9E-04 |
| hsa-miR-3913-5p | 2.26 | 5.0E-04 | 2.1E-03 |
| hsa-miR-556-5p | 2.31 | 5.1E-05 | 3.3E-04 |
| hsa-miR-484 | 2.32 | 2.9E-09 | 5.1E-08 |

| | | | |
|---|---|---|---|
| hsa-miR-103a-2-5p | 2.34 | 1.1E-04 | 6.3E-04 |
| hsa-miR-18b-3p | 2.36 | 1.1E-03 | 3.8E-03 |
| hsa-miR-610 | 2.37 | 1.0E-03 | 3.6E-03 |
| hsa-miR-196b-5p | 2.42 | 5.0E-07 | 5.8E-06 |
| hsa-miR-144-3p | 2.43 | 1.8E-08 | 2.9E-07 |
| hsa-miR-16-1-3p | 2.46 | 1.9E-04 | 9.7E-04 |
| hsa-miR-542-5p | 2.47 | 1.9E-04 | 9.8E-04 |
| hsa-miR-25-3p | 2.49 | 1.1E-12 | 9.6E-11 |
| hsa-miR-582-3p | 2.50 | 2.4E-05 | 1.6E-04 |
| hsa-miR-942 | 2.51 | 6.2E-09 | 1.0E-07 |
| hsa-miR-618 | 2.52 | 7.1E-04 | 2.8E-03 |
| hsa-miR-5582-3p | 2.53 | 8.2E-04 | 3.1E-03 |
| hsa-miR-3200-5p | 2.53 | 1.1E-04 | 6.3E-04 |
| hsa-miR-3913-3p | 2.54 | 2.8E-05 | 1.9E-04 |
| hsa-miR-3680-5p | 2.56 | 1.4E-04 | 7.9E-04 |
| hsa-miR-939 | 2.58 | 3.0E-04 | 1.4E-03 |
| hsa-miR-5010-5p | 2.58 | 2.9E-04 | 1.4E-03 |
| hsa-miR-4504 | 2.59 | 6.3E-04 | 2.5E-03 |
| hsa-miR-486-5p | 2.63 | 1.8E-12 | 1.3E-10 |
| hsa-miR-486-3p | 2.63 | 1.9E-12 | 1.3E-10 |
| hsa-miR-93-5p | 2.63 | 1.5E-11 | 7.1E-10 |
| hsa-miR-542-3p | 2.63 | 5.7E-08 | 8.2E-07 |
| hsa-miR-4532 | 2.65 | 4.1E-04 | 1.8E-03 |
| hsa-miR-941 | 2.68 | 1.7E-10 | 6.5E-09 |
| hsa-miR-182-5p | 2.70 | 6.8E-10 | 1.7E-08 |
| hsa-miR-183-5p | 2.71 | 1.5E-09 | 3.0E-08 |
| hsa-miR-4446-3p | 2.72 | 4.8E-04 | 2.0E-03 |
| hsa-miR-5094 | 2.76 | 7.9E-04 | 3.0E-03 |
| hsa-miR-296-5p | 2.84 | 4.7E-06 | 3.9E-05 |
| hsa-miR-1294 | 2.84 | 8.8E-07 | 9.3E-06 |
| hsa-miR-324-5p | 2.86 | 2.5E-09 | 4.7E-08 |
| hsa-miR-3158-5p | 2.99 | 2.2E-07 | 2.8E-06 |
| hsa-miR-5189 | 3.03 | 4.4E-04 | 1.9E-03 |
| hsa-miR-4492 | 3.03 | 1.2E-06 | 1.2E-05 |
| hsa-miR-3200-3p | 3.05 | 4.2E-05 | 2.8E-04 |
| hsa-miR-4508 | 3.09 | 7.0E-07 | 7.6E-06 |
| hsa-miR-1306-3p | 3.16 | 1.9E-07 | 2.4E-06 |
| hsa-miR-2115-5p | 3.17 | 1.1E-04 | 6.3E-04 |
| hsa-miR-4511 | 3.19 | 9.0E-05 | 5.3E-04 |
| hsa-miR-3688-5p | 3.19 | 1.5E-10 | 6.2E-09 |
| hsa-miR-3136-5p | 3.20 | 2.9E-03 | 9.2E-03 |
| hsa-miR-3158-3p | 3.20 | 3.2E-08 | 4.7E-07 |
| hsa-miR-3677-3p | 3.24 | 1.0E-03 | 3.7E-03 |
| hsa-miR-3688-3p | 3.31 | 4.1E-10 | 1.2E-08 |
| hsa-miR-3656 | 3.32 | 5.2E-10 | 1.4E-08 |
| hsa-miR-4637 | 3.47 | 2.6E-04 | 1.3E-03 |
| hsa-miR-4503 | 3.70 | 1.5E-06 | 1.5E-05 |
| hsa-miR-143-3p | 3.87 | 2.0E-15 | 2.8E-13 |

*$Log_2$ FC (fold change) represents ratio to miRNA levels in health subjects miRAN WITH ($log_2|FC| \leq 1.5$, FDR$\leq$0.01) were listed.




**Supplementary Figure 1.** Hierarchical cluster analysis demonstrates that a panel of 4 miRNAs: miR-375, miR-100-5p, miR-151a-3p, miR-25-3p could clearly distinguish EAC cancer cases from controls ($P<0.05$, Hotelling *T* test), normal (N) is for each healthy control, and tumor (P) is for each EAC patient. Higher copy number relative to the mean of all samples is expressed as red, and lower is expressed as green.