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Global analysis of serum microRNAs as potential biomarkers for lung adenocarcinoma

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Early diagnosis and the ability to predict the most relevant treatment option for individuals is essential to improve clinical outcomes for non-small cell lung cancer (NSCLC) patients. Adenocarcinoma (ADC), a subtype of NSCLC, is the single biggest cancer killer and therefore an urgent need to identify minimally invasive biomarkers to enable early diagnosis. Recent studies, by ourselves and others, indicate that circulating miRNAs have potential as biomarkers. Here we applied global profiling approaches in serum from patients with ADC of the lung to explore if miRNAs have potential as diagnostic biomarkers. This study involved RNA isolation from 80 sera specimens including those from ADC patients (equal numbers of stages 1, 2, 3, and 4) and age- and gender-matched controls ($n = 40$ each). Six hundred and sixty-seven miRNAs were co-analyzed in these specimens using TaqMan low density arrays and qPCR validation using individual miRNAs. Overall, approximately 390 and 370 miRNAs were detected in ADC and control sera, respectively. A group of 6 miRNAs, miR-30c-1* (AUC = 0.74; $P < 0.002$), miR-616* (AUC = 0.71; $P = 0.001$), miR-146b-3p (AUC = 0.82; $P < 0.0001$), miR-566 (AUC = 0.80; $P < 0.0001$), miR-550 (AUC = 0.72; $P = 0.0006$), and miR-939 (AUC = 0.82; $P < 0.0001$) was found to be present at substantially higher levels in ADC compared with control sera. Conversely, miR-339-5p and miR-656 were detected at substantially lower levels in ADC sera (co-analysis resulting in AUC = 0.6; $P = 0.02$). Differences in miRNA profile identified support circulating miRNAs having potential as diagnostic biomarkers for ADC. More extensive studies of ADC and control serum specimens are warranted to independently validate the potential clinical relevance of these miRNAs as minimally invasive biomarkers for ADC.

Introduction

Lung cancer is the leading cause of cancer death worldwide and the third most common cause of death from all causes. In 2010, in the United States alone, 222 520 new cases of lung cancer were diagnosed and 157 300 people died from this disease.[1] Approximately 85–90% of all cases of lung cancer are non-small cell lung cancer (NSCLC).[2] Until recently, NSCLC was treated as a single disease despite recognition of its molecular and histological heterogeneity.[3] NSCLC includes adenocarcinoma (ADC), squamous cell carcinoma, and large cell carcinoma. Recent reports indicate ADC to account for up to 50% of lung cancers.[4] Efficacy and safety results from recent clinical trials have shown the importance of sub-grouping NSCLC into its subtypes to achieve maximum benefit while minimising toxicity for patients[3,5] as, unfortunately, in light of this, there is merit in considering subtype when aiming to identify biomarkers.

Despite the devastating problem of NSCLC and the estimated 51% increased numbers of cases of this disease since 1985,[4] a panel of reliable serum biomarkers has not yet been identified. Existing lung cancer protein biomarkers include tumor-liberated proteins such as CEA, NSE, TPA, chromogranin, CA125, CA19–9, and Cyfra 21–1. While these are the best options currently available in the clinic, they each have limitations as detailed by Tarro et al.[6]

The interest in circulating RNAs as biomarkers is rapidly increasing as their potential is being realized. In 2008, we published the first whole genome microarray analysis indicating that many hundred mRNAs can be detected in serum.[7] More recently, ourselves and others have published data supporting a role for circulating miRNAs in a range of cancer types including breast,[8,9] prostate,[10,11] liver,[12,13] gastric,[14] and brain cancers.[15] Furthermore, number of recent studies of NSCLC specimens has supported the relevance of circulating miRNAs in NSCLC.[16-23] Advancing on our earlier work, and supported by the important data reported in the NSCLC serum studies performed by others, we present what we believe to be the largest global analysis of miRNAs (667 miRNAs) in serum specifically focusing on the most common type of NSCLC, adenocarcinoma.

Results

Patient characteristics

This study involved analysis of 80 serum specimens, including 40 sera from patients (22 male and 18 female) with ADC and

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Table 1. Adenocarcinoma patients and healthy controls

miRNA	Controls (y; mean ± SD)	ADC (y; mean ± SD)	*P* value
Stage 1 (6 male; 4 female)	59.9 ± 2.0	62.1 ± 2.0	0.450
Stage 2 (6 male; 4 female)	67.6 ± 40	68.9 ± 3.5	0.810
Stage 3 (5 male; 5 female)	58.2 ± 2.4	60.2 ± 3.2	0.630
Stage 4 (5 male; 5 female)	70.9 ± 2.0	70.0 ± 2.7	0.790

Table 2. Assessment of 5 miRNAs commonly detected in serum or plasma

miRNA	Control (mean C_T)	ADC (mean C_T)	*P* value
miR-16	21.5 ± 1.5	22.2 ± 2.9	0.748
miR-103	28.7 ± 1.6	30.1 ± 2.2	0.351
miR-93	26.5 ± 1.1	27.7 ± 3.1	0.604
miR-192	29.5 ± 0.9	29.9 ± 2.5	0.772
miR-451	25.0 ± 1.8	26.4 ± 4.2	0.640

40 age-, gender-, and BMI-matched healthy volunteers. **Table 1** summarizes the gender balance and age following sub-division of the matched specimens based on ADC stage at which the patients presented (see **Table S1** for data relating to each serum specimens).

RNA yield and miRNA presence

Total RNA quantification from each serum specimen showed the yields to be similar from the patient and control cohort. Specifically for each 250 μL of patient serum, an average of 1.88 ± 0.33 μg RNA was retrieved, with control sera producing a mean of 1.83 ± 0.2 μg RNA ($P = 0.98$).

The results from this study of 667 miRNAs showed that the numbers of miRNAs present in ADC and control sera do not differ substantially. Assuming C_T values of < 35 as indicative of miRNA presence, 230 ± 51 miRNAs were detected in serum from ADC patients and 240 ± 21 were detected in control sera ($P = 0.729$). Applying less stringent C_T values of < 40 as present, 326 ± 68 miRNAs were detected in patients sera and 336 ± 36 in control sera ($P = 0.759$).

Assessing for miRNAs reported to generally be present in serum or plasma

A number of miRNAs have been reported as typically present in serum/plasma including miR-16, miR-103, miR-93, miR-192, and miR-451. As expected, we found these miRNAs to be present in all specimens analyzed (**Table 2**).

miRNAs identified as associated with ADC using TaqMan low density arrays

TLDA data showed 3 miRNAs to be undetectable (assuming no amplification by 40 C_T to indicated absence) in all 40 control sera specimens, and present in ADC sera at all stages of disease. These include miR-566 and miR-939. A number of other miRNAs, while present at low levels in some control sera, were found to be present at substantially higher levels in ADC sera compared with control when all data was normalized to mean C_T, prior to comparison on ADC C_T to control C_T values. Specifically, the mean fold increases for these miRNAs in ADC serum specimens compared with control sera were as follows: miR-517c (21.6-fold; range: 2.1- to 63.9-fold); miR-770-5p (15.8-fold; range: 2.0- to 36.6-fold); miR-605 (50.4-fold; range 1.2- to 143.3-fold); miR-212 (10.7-fold; range: 2.3- to 21.6-fold); miR-601 (7.8-fold; range: 3.1- to 13.2-fold). Conversely, two miRNAs were found to be at substantial higher levels across the 40 normal sera specimens compared with ADC sera i.e., miR-656 (22.8-fold; range: 2.8- to 44.5-fold) and miR-339-5p (21.4-fold; range: 4.8- to 69.1-fold) were detected at higher levels in control compared with ADC serum specimens.

qPCR validation of results arising from TLDA analysis

Array technology enabled co-analysis of many (667) miRNAs. However, in order to establish if the results from such analysis would consistently be found using a more routine technique that could potentially be translated to hospital laboratories for analysis, 8 miRNAs were selected for individual analysis in all 80 specimens using standard quantitative polymerase chain reaction (qPCR) analysis. This more limited group of miRNA was selected as RNA quantities available were limited. However, these would prove in principle if validation would be achieved. The fact that little, if any, information is published on these miRNAs means that their selection also adds to the advancement of our understanding of miRNAs. Specifically, these miRNAs included miR-566, miR-550, and miR-939 [found by TLDAs to be absent from control sera ($n = 40$) and present in ADC sera ($n = 40$)]. The other 3 miRNAs found to be increased in ADC and so selected for qPCR analysis were miR-616*, miR-146b-3p, and miR-30c-1*, which we had previously identified as potential biomarkers for ADC but in a more limited pilot study of stage 1 ADC only ($n = 10$) and age- and gender-matched control ($n = 10$) sera (**Sup. Data 1**). The fact that this trend was also found through the TLDA analysis here, i.e., miR-616*, miR-146b-3p, and miR-30c-1* were present (<35 C_T) in stage 1, but were absent from matched control sera supported their further investigation. The other two miRNAs selected for assessment by qPCR were miR-339-5p and miR-656, that we identified as at substantially lower levels in ADC sera compared with controls specimens.

miR-566

Using quantitative PCR analysis, miR-566 was detected in all specimens with the exception of one ADC specimen. Directly comparing each ADC and matched control showed miR-566 to be 70 ± 29.4-fold increased in ADC sera, in all but 5 matched pairs (**Fig. 1A**). The AUC value from ROC analysis was 0.80, demonstrating a significant ($P = 0.0001$) difference between ADC patients and healthy controls (**Table 3**). As individual matched normal specimens would not necessarily always be available for comparison, we also analyzed levels in each ADC specimen compared with the overall mean levels in the 40 controls; showing a 19.1 ± 4.4-fold increase in 95% of cases (**Fig. 1B**). Considering the 4 stages of ADC, levels of circulating serum miR-566 in ADC specimens (compared with their individual matched control pairs) were found to increase in stage 2 disease compared with stage 1. However, levels in stage 3 decreased substantially compared with stage 2 before increasing again in stage 4 disease

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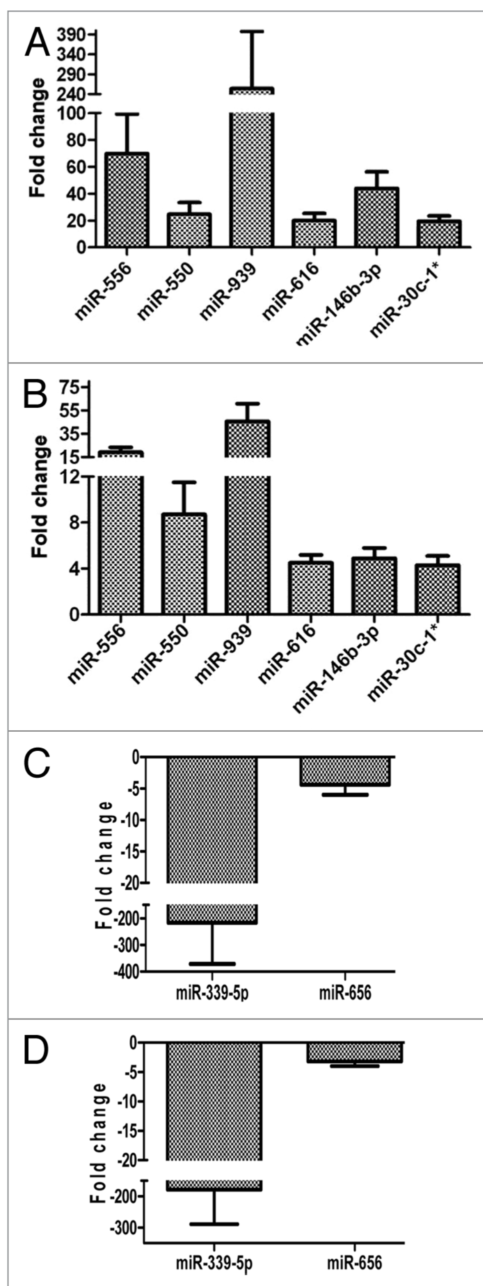

Figure 1. miR-556, -550, -939, -616*, 146b-3p, and -30c-1* were detected at substantially higher amounts in serum from ADC patients (*n* = 40) compared with their individually (**A**) or mean value (**B**) for their paired age- and gender-matched controls (*n* = 40). miR-339-5p and miR-656 were detected at substantially lower levels in serum from ADC patients (*n* = 40), as shown after comparing their individual (**C**) or mean value (**D**) for their paired age- and gender-matched controls (*n* = 40). Graphs represent fold changes in ADC (mean ± SE).

(**Fig. 2**). This trend was also observed when miR-566 in individual ADC sera was compared with the mean level in control specimens (**Fig. 3**).

miR-550

miR-550 was detected in 100% of ADC sera. In 15% of comparison pairs (6/40) miR-550 went from undetectable in normal serum to present in ADC. While some level of miR-550 was detectable in 34 of the normal sera, the amounts were substantially greater in ADC compared with control sera in the majority (75%) of cases; with an average fold increase of miR-550 in ADC sera of 24.6 ± 8.8 (**Fig. 1A**), when compared with its matched control or 8.7 ± 2.8 when compared with the mean of the controls (**Fig. 1B**). For miR-550, the AUC value from ROC analysis was 0.72, showing a significant (P = 0.0006) difference between ADC patients and healthy controls (**Table 3**). When considering age- and gender-matched pair comparisons, serum levels of miR-550 increased in stage 2 disease compared with stage 1, with levels in stage 3 decreasing substantially compared with stages 1 and 2, before increasing again in stage 4 disease (**Fig. 2**). Comparison of each ADC with the mean of control values indicated a marginal increase from stage 1 to stage 2 to stage 3, with an apparently more substantial increase at stage 4 (**Fig. 3**). However, it should be noted that this increase is strongly influenced by one stage 4 ADC serum specimen that had exceptionally high levels of miR-550. Eliminating this specimen bring the average fold increase in stage 4 to a similar level to that in stages 1–3 inclusively.

miR-939

miR-939 was detected in 100% of serum specimens and was found to be at substantially higher level (254.2 ± 143.4-fold) in 85% of cases where ADC specimens were compared directly to their age- and gender-matched control sera (**Fig. 1A**). The AUC value from miR-939 ROC analysis was 0.82, demonstrating a significant (P = 0.0001) difference between ADC patients and healthy controls (**Table 3**). Comparison of each ADC specimen to the mean level of miR-939 in control sera showed an average increase in ADC of 45.6 ± 15.2-fold (**Fig. 1B**). Of note, the same levels of miR-939 were detected in one ADC specimen when compared with its matched control levels, while 3 (stage 3) sera specimens had slightly lower levels of miR-939 compared with control, reflecting a mean difference of 1.7 ± 0.5 C_T. Considering the 4 disease stages, both matched-pair comparisons and comparisons of individual ADC specimen levels with the mean control level showed levels of circulating serum miR-939 increased in Stage 2, with levels in Stage 3 decreasing substantially compared with Stages 1 and 2, before increasing again in Stage 4 disease (**Figs. 2 and 3**).

*miR-616**

miR-616* was detected in 98% of ADC serum specimens. In 30% of matched specimens, miR-616* went from undetectable in controls to being present in ADC. While miR-616* was within detectable levels in 27 of control sera, in the majority (82.5%) of matched specimens, the amounts were substantially higher level (20 ± 5.2-fold) in ADC compared with individual paired control sera (**Fig. 1A**). The miR-616* AUC value from ROC analysis was 0.71, demonstrating a significant (P = 0.001) difference between ADC patients and healthy controls (**Table 3**). Compared with mean of controls, the increased levels of miR-616* in ADC was found to be 4.5 ± 0.7-fold (**Fig. 1B**). Levels of miR-616* detectable in ADC serum did not consistently correlate with disease Stage (**Figs. 2 and 3**).

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miR-146b-3p

miR-146b-3p was detected in 95% of ADC serum specimens. In 51.5% of matched specimens compared, it went from undetectable in controls to being present in ADC. In 5% of cases this miRNA was absent from both the ADC and its matched control specimen. Where miR-146b-3p was detected in both ADC and control sera, the general trend was substantially higher levels (44 ± 12.3-fold) in ADC compared with age- and gender-matched control sera (**Fig. 1A**). For miR-146b-3p, the AUC value from ROC analysis was 0.82; demonstrating a significant (P = 0.0001) difference between ADC patients and healthy controls (**Table 3**). This was reflected as 4.9 ± 0.9-fold when comparing individual ADC specimens that showed increased levels of miR-146b-3p to the average levels in the controls (**Fig. 1B**). Considering the 4 stages of ADC, as for miR-566, levels of circulating miR-146b-3p increased in Stage 2 disease compared with Stage 1. However, levels in Stage 3 and 4 decreased compared with Stage 2 (**Figs. 2 and 3**).

*miR-30c-1**

miR-30c-1* was detected, by qPCR, in 70% of ADC serum specimens and in 28% of control sera. In 53% of cases, miR-30c-1* went from undetectable in controls to being present in ADC. When miR-30c-1* were detected in control serum, in general the amounts present were substantially higher (19.5 ± 3.9-fold) in early stage ADC compared with their respective matched controls. Of note, in a limited number of matched pairs (15%; 6/40) lower levels of miR-30c-1* were found in ADC compared with matched control sera. Overall, however, the AUC value from miR-30c-1* ROC analysis was 0.74 demonstrating a significant (P = 0.00018) difference between ADC patients and healthy controls (**Table 3**). Comparing increased levels of miR-30c-1* in each ADC sera specimen, a mean increase of 4.3 ± 0.8 was found compared with the average in controls. Again a minority (12.5%) of ADC specimens showed lower levels (2.1 ± 0.5) of this miRNA compared with matched controls in early disease. Considering the 4 disease stages, as for a number of other miRNAs evaluated, miR-30c-1* levels increase in stage 2 disease compared with stage 1, with levels in stage 3 decreasing compared with stage 2, before increasing again in stage 4 disease (**Figs. 2 and 3**). Importantly, while miR-30c-1* was detectable in only 70% of ADC specimens overall, its absence was restricted to the earlier stages of the diseases and, importantly, miR-30c-1* was detected in 100% of stage 4 specimens.

miR-339-5p

qPCR analysis confirmed that the levels of miR-339-5p were substantially lower in serum from ADC patients compared with that from healthy controls (**Fig. 1C**). Considering the individual stages of disease, miR-339-5p was substantially lower in 40% and 70% of the stage 1 and stage 2, respectively, and in 100% of both stage 3 and stage 4 ADC serum specimens. The AUC value from miR-339-5p ROC analysis was determined to be 0.6 (**Table 3**).

miR-656

qPCR analysis also validated our TLDA analysis of miR-656 i.e., miR-656 level was down in ADC serum specimens compared with their age- and gender-matched control sera (**Fig. 1D**). This was found to be the situation in 40% of stage 1 specimens, 60% of stage 2 specimens, and 70% of both stages 3 and 4. The AUC value from miR-656 ROC analysis was 0.6 (**Table 3**).

Table 3. AUC value from ROC analysis

Upregulated	AUC	*P* value
miR-566	0.79	0.0001
miR-550	0.72	0.0006
miR-939	0.82	0.0001
miR-616*	0.81	0.0001
miR-146b-3p	0.71	0.001
miR-30c-1*	0.74	0.0002
Downregulated	**AUC**	***P* value**
miR-339-5p	0.6	0.1
miR-656	0.6	0.2

Co-analysis of panel of miRNAs in all specimens

As all 6 miRNAs identified as potential panel members (based on being increased in ADC sera vs. that in serum from healthy controls) were not overexpressed in 100% of ADC specimens, we co-assessed their expression. A minimum of 2 miRNAs and up to the maximum of all 6 miRNAs were overexpressed in any given ADC specimen. This emphasizes the relevance of assessing all 6 miRNA. Considering all 6 miRNAs, the AUC value from ROC co-analysis was 0.7, indicating a significant (P < 0.0001) difference between ADC patients and healthy controls. As shown in **Figure 4A**, co-analysis of the miRNAs show a 13.8 ± 2.9-fold increase levels in ADC compared with control sera. Considering each stage of disease individually, this was reflected in their increased levels in stage 2 compared with stage 1, with reduced levels in stage 3 sera before increasing again in stage 4. In the relation to the combination of two miRNAs (miR-339-5p and miR-656) reduced in ADC sera, the AUC value from ROC co-analysis was 0.6, indicating a significant (P = 0.02) difference between ADC patients and healthy controls. As shown in **Figure 4B**, co-analysis of the two miRNAs show 110.7 ± 77.7-fold decrease in levels in ADC compared with control sera. Considering each stage of disease individually, this was reflected in their decreased levels from stage 1 to stage 2 to stage 3, with no substantially difference noted between stage 3 and stage 4.

Bioinformatics analysis

Pathway analysis was undertaken for all miRNA differentially regulated in ADC compared with normal sera using DIANA miRPath. This software was able to identify all the mRNAs targeted by our differentially regulated miRNA and finally known KEGG pathways associated with them and P value (**Table S2**). Interestingly, among the statistically significant pathways, we found pathways associated with cancer, various signaling pathways including Hedgehog signaling pathway, Wnt signaling pathway, and TGF-β signaling pathway (**Table S2**). Association of these differentially-regulated miRNA with range of tumors were also studied using publically available data mined from GEO (**Table S3**). GEO accession numbers were identified and

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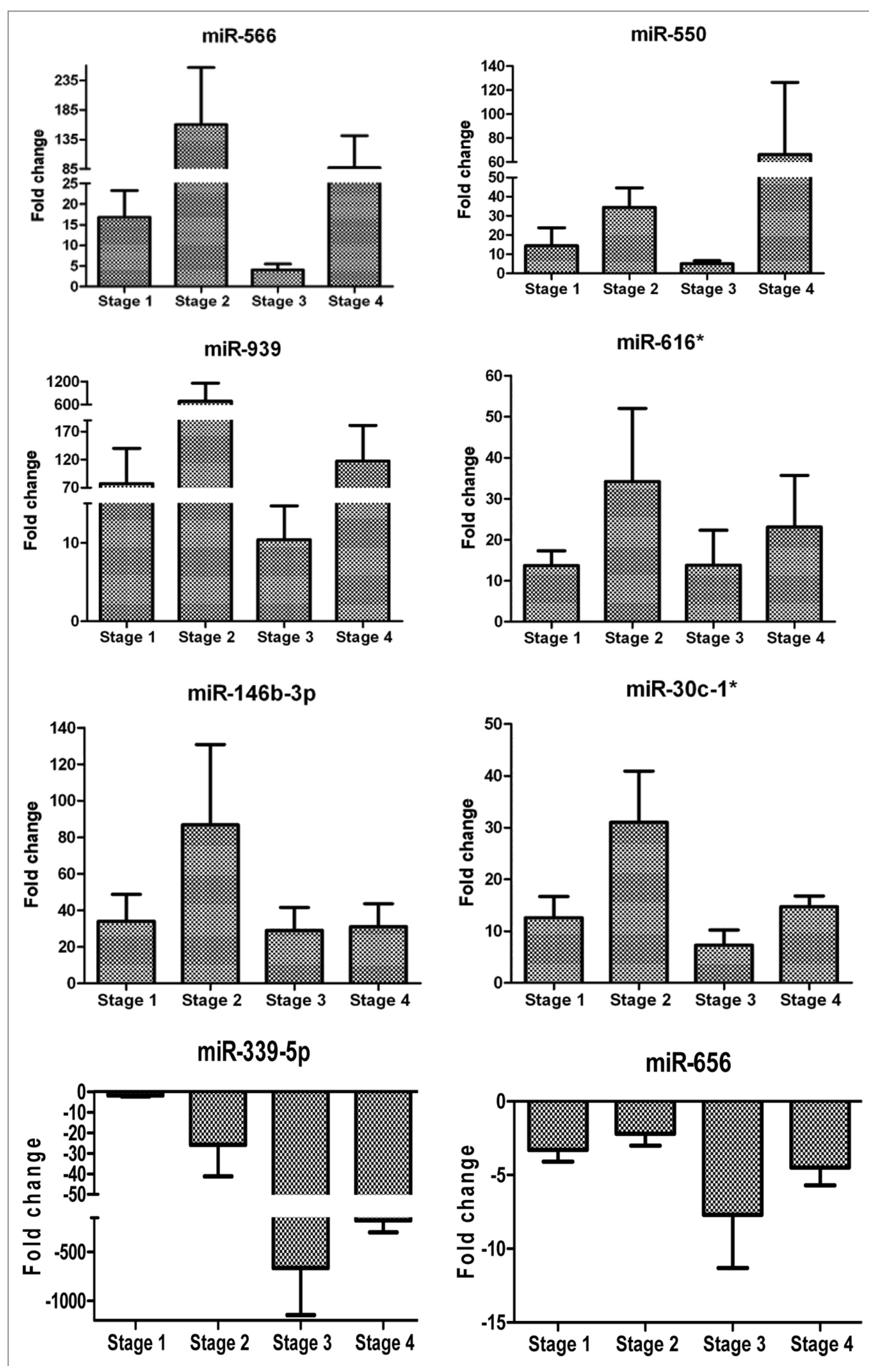

Figure 2. Considering ADC tumor stages, miR-556, -550, -939, -616*, 146b-3p, and -30c-1* were detected at substantially higher amounts in serum from stage 1 ADC patients ($n = 10$) compared with their individually paired age- and gender-matched controls. The circulating amounts of each of these miRNAs increased significantly again in stage 2 compared with stage 1, before decreasing again in stage 3 disease and then increasing again, to some extent, in stage 4. Conversely, miR-339-5p levels were decreasing from stage 1 to stage 2 and then to stage 3 with a lesser effect from stage 3 to stage 4; although this was not significant. A similar trend was observed for miR-656 except that the reduced levels in stages 1 and 2 disease did not differ significantly from each other. Graphs represent fold changes in ADC compared with their individually paired age- and gender-matched controls (mean ± SE).

was analyzed with GEO2R to obtain log fold-change and *P* value (**Table S3**).

Discussion

ADC of the lung is currently the single biggest killer in cancer. Studies by us and others strongly support a potential role for RNAs as circulating minimally-invasive biomarkers. In fact, a number of recently published and emerging studies suggest that miRNAs exist in sera that are associated, in general, with non-small cell lung cancer. These include studies of between 5 and 467 miRNAs in plasma or serum assessing NSCLC overall in cohort sizes ranging from 11 to 400 specimens,[16,20,22-26] the largest study analyzing 91 miRNAs in 400 NSCLC specimens,[17] some of which are discussed in further detail below. In what we believe to be the initial study focusing on ADC, Rabinowits et al.[27] isolated exosomes from plasma of 27 patients and 9 controls. While the exosomal-contained miRNAs may not contain all circulating miRNAs, the study by Rabinowits et al.—along with the more general studies of circulating miRNAs in NSCLC mentioned above—indicate the importance and relevance of progressing to more global profiling discovery and subsequent validation to seeking circulating miRNAs as diagnostic, prognostic and/or predictive biomarkers.

Advancing on this, here we report what we believe to be the first large study (677 miRNAs) of circulating miRNAs specifically in ADC. Our study compared the miRNA profile of ADC with age- and gender-matched control sera. The main novel findings of this study include the observation that there are >300 miRNAs detectable in serum and although many (270–290) miRNAs are present in serum from healthy controls as well as ADC patients, a number of miRNAs are differentially detected (based on absent vs. presence or differential levels of detection) under these circumstances. Here we identified a group of 6 miRNAs that exist at substantially higher levels in the ADC compared with control sera. While the numbers of specimens from each disease stage are too limited to derive a meaningful statistical conclusion (40 total; $n = 10$ each for stages 1, 2, 3, and 4), the analysis frequently showed increased amounts of these miRNAs to be present in serum from patients with stage 2 disease compared with stage 1, with levels frequently reduced in stage 3 before rising again in stage 4. However, we are also very mindful of the limited numbers of specimens available for analysis at each disease

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stage and that the numbers involved prevent us drawing any firm conclusions from this observation. The good correlation overall between the TLDA analysis and the qPCR analysis on individual specimens is an important observation. It would not be feasible to globally profile miRNAs individually in all patients' specimens (huge cost implications, so unlikely to be relevant to many research laboratories and so the lung cancer research community as a whole). So we believe that evidence supporting the rationale for being able to pool specimens for global analysis and to realize the same trend for miRNAs when individually assessed by qPCR is relevant.

In relation to numbers of circulating miRNAs and considering relevant studies performed by others, Chen et al.[16] reported on an important study including analysis of serum from 7 young Chinese subjects where over 100 and 91 miRNAs, respectively, were detected in male and female subjects. Assessing cohorts of 30 NSCLC patients based on disease survival, Hu et al.[20] detected 109 and 101 miRNAs in the serum from patients with longer- and shorter-survival times, respectively. In the study reported here which included serum from 44 males and 36 females, we did not find any association between miRNA numbers and gender. Incidentally, this is in agreement with a recent study by Heegaard et al.,[18] where no association was found between gender and serum/plasma miRNA profiles. However, compared with the study by Chen et al.,[16] we detected many more sera miRNAs overall, i.e., approximately 390 and 370 miRNAs in ADC and control sera, respectively. The greater number of miRNAs detected here may be due to a combination of factors, including advancement on technology for miRNAs identification and evaluation—and so the numbers of miRNAs known to exist and detectable—as well as that somewhat larger cohorts of cases possible for us to evaluate. Of note, Heegaard et al.[18] reported considerable difference in miRNA levels (with 14 miRNAs significantly reduced in serum from African American compared with European Americans) so it is conceivable that, as with many genetic and phenotypic traits associated with cancer, race may some way contribute to circulating miRNAs profiles; emphasizing the importance of increasing the numbers of international collaborative studies in this field. Overall, we believe that our work complements studies by Chen et al.[16] and Hu et al.,[20] collectively adding to our understanding of the numbers and scope of miRNAs in circulation.

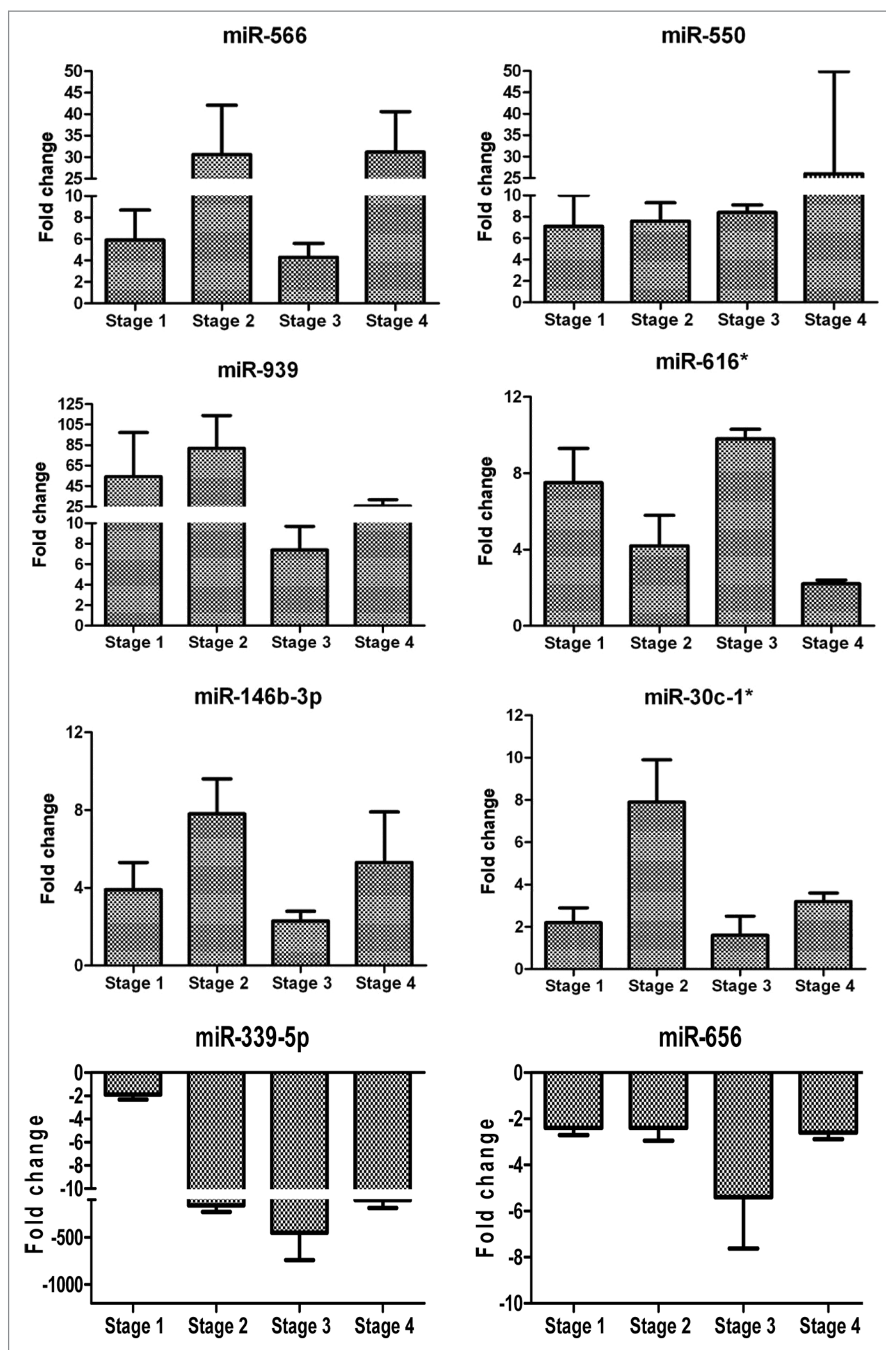

Figure 3. Considering ADC tumor stages, miR-556, -550, -939, -616*, 146b-3p, and -30c-1* were detected at substantially higher amounts whereas, miR-339-5p and miR-656 was downregulated in serum from all stages of ADC patients compared with the mean detection level in the paired age- and gender-matched controls; although a direct association was not found with disease stage. Graphs represent fold changes in ADC compared with their individually paired age- and gender-matched controls (mean ± SE).

In relation to disease biomarkers, assessing NSCLC overall as a single disease, Chen et al.[17] evaluated 91 miRNAs and identified 10 of these as potential biomarkers for NSCLC. Importantly their study did not include analysis of the

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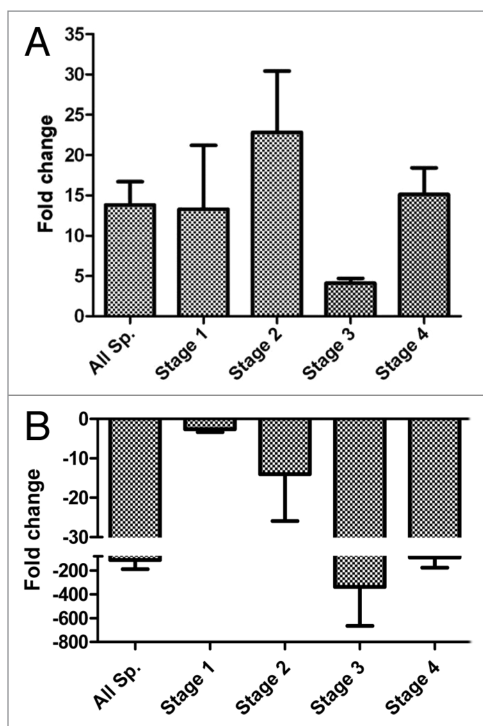

Figure 4. Co-analysis of miR-556, -550, -939, -616*, 146b-3p, and -30c-1* shows significantly increased levels in ADC sera overall compared with their collective levels in paired age- and gender-matched controls. Increased levels of these 6 miRNAs were found in Stage 2 sera compared with that in Stage 1, but fell again in Stage 3 before rising in Stage 4 (**A**). Co-analysis of miR-339-5p and miR-656 showed reduced levels in ADC sera overall compared with their combined levels in paired age- and gender-matched controls (**B**). Graphs represent fold increase in ADC compared with the mean levels in control sera (mean ± SE).

6 miRNAs (miR-30c-1*, miR-616*, miR-146b-3p, miR-566, miR-550, and miR-939) that we detail in this study of ADC. Of the 10 miRNAs reported as differentially expressed,[17] miR-199a-5p was found to be substantially (15.64-fold) increased in NSCLC compared with control sera. In keeping with this, we found miR-199a-5p to be present in ADC sera but absent from control sera. The remaining 9 miRNAs reported by Chen et al.[17] were not substantially different in our ADC and control sera. Differences between these two observations are likely to be contributed to by the fact that our study was specifically related to ADC, while Chen et al.[17] reported on NSCLC in general but they did not give consideration to the different subtypes of NSCLC (including ADC).

Assessing 30 serum miRNAs in NSCLC compared with controls, Heegaard et al.[18] observed reduced quantities of 7 miRNAs including miR-221, let-7a, -155, 17-5p, -27a, -106a, and -146b. Interestingly our analysis showed a similar trend for miR-221, let-7a, 17–5p, -27a, and -106a.

For miR-155, we observed increased levels in stage 1 disease, but reduced levels for stages 2–4 inclusively (and so we did not consider this to be one of the most relevant miRNAs from our study). The discrepancy with miR-155 between the study by Heegaard et al. and the work presented here may, again, be attributed to the disease being analyzed (NSCLC collectively vs. ADC) and the stage of disease, i.e., Heegaard et al.[18] included stages 1 and 2 of NSCLC, while we considered all 4 stages of ADC. Of note, in a study of serum from 35 lung cancer patients (including 18 small cell lung cancers and 9 NSCLC, but the subtypes were not defined), Roth et al.[21] reported levels of miR-155 to be significantly higher in lung cancer compared with benign disease.

Our data on miR-146b conflicted with that found by Heegaard et al., i.e., miR-146b levels were substantially increased in our ADC but reduced in the NSCLC analyzed by Heegaard et al. However, as Heegaard et al. correctly outline, this miRNA has previously been reported as increased in cancerous tissue[28,29] supporting its likelihood to be increased as we observed in ADC serum, rather than decreased in serum from cancer patients. Recently Malleter et al.[30] also reported overexpression of miR-146b in NSCLC-N6 cells and associated with carcinogenesis of lung cancer. A panel of 6 miRNA classifiers for predicting recurrence in stage 1 lung cancer was reported that included miR-30c-1* and miR-146b-3p.[31,32]

Multiple signaling pathways were affected by our differentially-regulated miRNA including Hedgehog signaling pathway, Wnt signaling pathway, and TGF-β signaling pathway. These pathways have been associated with lung carcinogenesis (Wnt signaling pathway),[33] cell invasion (TGF-β signaling pathway),[34] and maintaining progenitor cells in lung cancer (Hedgehog signaling pathway).[35]

Conclusion

In what we believe to be the first reported global profiling of serum from ADC and control patients, we report many hundred miRNAs to exist in the circulation. Furthermore, from this analysis we have identified 6 miRNAs at substantially higher levels—and 2 miRNAs at substantially lower levels—in ADC sera compared with control; which could potentially contribute to a panel of minimally-invasive circulating biomarkers for ADC diagnosis. Breaking down the cohort of ADC specimens into disease stages showed a tendency for increased levels particularly in earlier Stages of disease; suggesting that they may have particular relevance for early diagnosis. However, as numbers of specimens available for each stage analysis was limited, this observation in relation to tumor stages would need to be advanced to larger cohort to assess this further. While independent validation in much larger cohorts (including both A and B tumor sub-stages), we believe that the data presented in this study adds novel information to this field of circulating miRNAs and the quest to identify biomarkers for diagnosis and, ultimately, more personalized management of cancer patients.

Material and Methods

Patient characteristics

The study involved analysis of 667 miRNAs in 80 serum specimens. Forty specimens were procured from consenting patients who were diagnosed with adenocarcinoma (ADC) of

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the lung. Serum specimens from 40 age-, gender-, and BMI-matched healthy volunteers were analyzed as controls. Ethics approval was obtained for these studies and all patients gave informed consent.

RNA extraction

RNA was isolated from 250 μL of each serum specimen, after passing it through a 0.45 μm-filter. Specifically, RNA was extracted with TriReagent (Sigma, T9424) using a modification of the procedure that we previously reported.[7] RNA was subsequently assessed at 230, 260, and 280 nm using a Nanodrop ND-1000 (Labtech International).

Global analysis of miRNAs

Global profiling of miRNA expression was performed using TaqMan low density arrays (TLDAs; Applied Biosystems (4398965, 4398966) representing 667 miRNAs on 2 array card/TLDA panel A (377 miRNAs) and panel B (290 miRNAs). cDNA was prepared from 3 μL RNA (25 ng/μL) according to the ABI microRNA TLDA Reverse Transcription Reaction protocol. The cDNA product (2.5 μL per specimen) was pre-amplified according to the ABI TLDA pre-amplification protocol (Applied Biosystems; 4399233, 4399201). The amplified product was then quantified using an Applied Biosystems 7900 HT Real-Time PCR system. For initial screening, pooled specimens (equal quantities) of RNA for each cancer stage vs. pooled specimens of each set of matched controls were evaluated. Subsequent to the success of this step, individual specimens were analyzed.

Real-time quantification of micro-RNAs

Validation of miRNAs by single and co-analysis was performed using qPCR analysis (Applied Biosystems). This assay includes a reverse transcription (RT) step using the TaqMan microRNA reverse transcription kit. Briefly, the RT reaction consisted of 1.5 μL 10× RT Buffer, 0.15 μL dNTPs 100 mM, 0.19 μL RNase Inhibitor 20 U/μL, 1.0 MultiScribe reverse transcriptase, 3 μL of primer, and 5 ng total RNA in a final volume of 15 μL. The reaction was then incubated in using a 7900 HT Real-Time PCR system for 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C, and then held at 4 °C. The RT products were subsequently amplified with sequence-specific primers using the Applied Biosystems 7900 HT Real-Time PCR system. The 20 μL PCR mix contains 1.33 μL RT-product, 1 μL TaqMan Universal PCR Master Mix (20×), 1 μL TaqMan probe. The reactions were incubated in a 96-well plate at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and at 60 °C for 1 min.

Data analysis

The ABI TaqMan SDS v2.3 software was utilized to obtain raw C_T values. As each TLDA was performed for a given specimen ($n = 80$) based on fixed, constant quantities of RNA in each case, to avoid introducing any bias at this stage, the raw C_T data (SDS file format) were exported from the Plate Centric View. For analysis of TLDA data, values for each specimen were normalized to the mean of the C_T values. Fold changes in ADC serum vs. control serum were thus determined by the ΔC_T method as described previously, i.e., cycle threshold (C_T) ADC − (C_T) control.[16,19,20,36] Excel and SPSS 16.1 statistics packages were used. To assess sensitivity and specificity, receiver operating characteristic (ROC) curves were created using GraphPad.

Bioinformatics analysis

Web-based computational tool DIANA miRPath v2.0 was used to identify potential altered molecular pathways and gene transcript by the expression of each miRNA.[37] FDR correction was used for all miRNA except miR-146b-3p that enables false discovery rate correction (Benjamini and Hochberg) to the resulting significance level. microT-CDS was used for pathway enrichment analysis that provide higher sensitivity and uses only the 3′-UTR target sites. We also identified association of each miRNA with various tumors using data sets from GEO omnibus repository.

Disclosure of Potential Conflicts of Interest

The authors have no conflict of interest to declare.

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Supplemental Materials

Supplemental materials may be found here:
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