Current Problems in Cancer 44 (2020) 100540

Contents lists available at ScienceDirect

Current Problems in Cancer

journal homepage: www.elsevier.com/locate/cpcancer

Identification of differentially expressed circulating serum microRNA for the diagnosis and prognosis of Indian non–small cell lung cancer patients

Sachin Kumar[a,*], Surender K. Sharawat[a], Ashraf Ali[b], Vikas Gaur[a], Prabhat Singh Malik[a], Sunil Kumar[c], Anant Mohan[b], Randeep Guleria[b]

[a] *Department of Medical Oncology, Dr. B.R.A. Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi, India*
[b] *Department of Pulmonary Critical Care and Sleep Medicine, All India Institute of Medical Sciences, New Delhi, India*
[c] *Department of Surgical Oncology, Dr. B.R.A. Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi, India*

ABSTRACT

Purpose: Identification of noninvasive blood-based biomarkers is of utmost importance for the early diagnosis and predicting prognosis of advance stage lung cancer patients. MicroRNAs (miRNAs) has been implicated in numerous diseases, however, their role as diagnostic and prognostic biomarkers in Indian lung cancer patients has not been evaluated yet.
Methods: For the identification of differentially expressed miRNAs in the serum of non–small cell lung cancer (NSCLC) patients, we performed small RNA sequencing. We validated the expression of 10 miRNAs in 75 NSCLC patients and 40 controls using quantitative reverse transcription polymerase chain reaction (PCR). miRNA expression was correlated with survival and therapeutic response.
Results: We identified 16 differentially expressed miRNAs in the serum of NSCLC patients as compared to controls. We observed significant downregulation of miR-15a-5p, miR-320a, miR-25-3p, miR-192-5p, let-7d-5p, let-7e-5p, miR-148a-3p, and miR-92a-3p in the serum of NSCLC patients. The expression of miR-375 and miR-10b-5p was significantly downregulated in lung squamous cell carcinoma patients than controls.

☆ Acknowledgement: This work was supported by a funding from the Science & Engineering Research Board (SERB), Govt. of India, New Delhi (Grant No. SB/YS/LS-348/2013).
☆☆ Conflict of interest: None.
* Correspondence to: Sachin Kumar, Department of Medical Oncology, Dr. B.R.A. Institute Rotary Cancer Hospital, All India Institute of Medical Science, New Delhi-110029, India.
E-mail address: sksingla@aiims.ac.in (S. Kumar).

https://doi.org/10.1016/j.currproblcancer.2020.100540

The expression of miR-320a, miR-25-3p, and miR-148a-3p significantly correlated with stage. None of the miRNAs were correlated with survival outcome and therapeutic response.
Conclusions: We conclude that the relative abundance of miRNAs in serum may be explored for the development of miRNA-based assays for better diagnosis and prognosis of NSCLC. Moreover, further studies are warranted to elucidate the role of some of the less explored miRNAs, such as miR-375 and miR-320a, in the pathogenesis of NSCLC.

ARTICLE INFO

Keywords: MicroRNA; Lung cancer; Diagnosis; Prognosis; Survival

Introduction

Lung cancer accounts for 11.6% of all cancers and 18.4% of all cancer-related deaths making it the most common and deadly cancer globally.[1] Histologically, lung cancer is classified as either non–small cell lung cancer (NSCLC) or small cell lung cancer (SCLC). NSCLC is further classified as adenocarcinoma (LUAD) and squamous cell carcinoma (LUSC). The pathologic and genetic signatures of LUAD and LUSC are different with many targetable driver mutations, such as those in EGFR, KRAS, ALK, ROS1, BRAF has been identified in LUAD.[2-4] Despite targeted agents and immunotherapies, the survival of advanced stage NSCLC remains very poor. This is mainly because almost two-third of all lung cancer cases are diagnosed at locally advanced or advanced stage that cannot be treated effectively. Hence, better biomarkers are needed, particularly using noninvasive methods, for early diagnosis and predicting prognosis and therapeutic response.

One such interesting class of biomarkers which can be stably detected in various human body fluids is microRNA (miRNA). miRNAs are small, ≈18-22 nucleotides long, noncoding RNA, which are involved in post-transcriptional gene silencing mainly through complementary base-pairing to the 3′ untranslated region (UTR) of target mRNA, followed by degradation of target mRNA and/or translational inhibition.[5] Several miRNAs have been identified that regulate the expression of various genes involved in carcinogenesis.[6,7] A number of studies have shown aberrant expression of miRNAs in tumor tissues of several human cancers, including NSCLC.[8-11] Several studies have also evaluated the expression of miRNAs in various body fluids for their use as noninvasive diagnostic and prognostic biomarkers.[12] However, to the best of our knowledge only 2 blood-based miRNA panels have successfully progressed to clinical testing as complementary tests for low dose computed tomography (LDCT).[13,14] Hence, we designed this study for better understanding on the role of circulating serum miRNAs as diagnostic and prognostic biomarker as well as predictor of survival and therapeutic response. To this best of our knowledge, this is first report from India on expression profiling of serum miRNAs in NSCLC patients.

Materials and methods

Patients

In this prospective study, 75 newly diagnosed and primarily untreated patients of NSCLC and 40 cancer-free controls were enrolled from All India Institute of Medical Sciences, New Delhi, India during the years 2014-2018. Pretreatment assessment included evaluation of Eastern Cooperative Oncology Group performance status, radiography, and CT scan of the chest and upper abdomen. The staging was done using recommendations of the International Association for the Study of Lung Cancer Staging Committee for NSCLC.[15] In NSCLC patients who underwent chemotherapy for at least 3 cycles, response to therapy was classified according to RECIST v1.1

criteria.[16] The study was approved by the Institutional Ethics Committee and written informed consent was obtained from all the subjects.

Small RNA sequencing for global miRNA profiling

RNA was isolated from serum samples using miRNeasy Serum/Plasma kit (QIAGEN, Germany). A total of 10 subjects (4 NSCLC and 6 controls) were used for miRNA profiling to identify differentially expressed serum miRNAs in NSCLC. miRNA profiling was carried out with NxGenBio Life Sciences (Delhi, India) using illumina HiSeq 2000 platform. Total RNA was run on polyacrylamide gel for the purification of small RNAs of 18-36 bases. Purified RNA was ligated with adaptors (both 3′ and 5′), reverse transcribed using illumina small RNA reverse transcription primer and amplified by a 15-cycle PCR. PCR products were purified and quantified for sequencing. RNA 3′-adapter was specifically modified to target miRNAs that have a 3′-hydroxyl group resulting from enzymatic cleavage by Dicer or other RNA processing enzymes. Adapters were ligated to each end of the RNA molecule and a reverse transcription reaction was used to create single-stranded complementary DNA (cDNA). cDNA was PCR amplified using a common primer and a primer containing one of barcode index sequences. This cDNA library was then purified using AMpure beads and quality checked on Bioanalyzer 2100 (Agilent Technologies). Indexed small RNA libraries were multiplexed in equimolar amounts, denatured and loaded on flow-cell lanes for cluster generation. Using illumina TrueSeq v3 sequencing chemistry, indexed libraries were sequenced on the HiSeq 2000 with 50bp single end chemistry.

Raw data generated in FastQ format was analyzed using miRNAkey software where raw reads were first filtered for high quality reads (Phred quality score $>$ 20). Adaptor sequences are trimmed (Trimmomatic v. 0.36) and reads that fall between 18-24 bases were further used. Filtered high quality raw data was mapped on to Sanger miRBase v. 21 and known miRNAs were identified. Raw read mapping statistics for each miRNA was calculated, normalized, and compared between NSCLC and controls for the identification of differentially expressed miRNAs using DESeq. To identify differentially expressed miRNAs, we used the following thresholds: an absolute log2 fold change value of 1 (a $\geq$ 2-fold change in expression), or an adjusted *P* value [for differences in mean test)of 0.05, or both.

Quantification of miRNA using quantitative reverse transcription PCR (qRT-PCR)

The quantification of expression of various miRNAs was performed using miScript SYBRGreen PCR kit (QIAGEN, Germany). All the quantifications were performed on Roche LightCycler II instrument (Roche Diagnostics). All the samples were run in duplicates and *Caenorhabditis elegans* miR-39 was used as exogenous control (Ce_miR-39_1 miScript Primer Assay, QIAGEN, Germany). Samples without RNA template were used as negative controls. The relative quantification of miRNA expression levels were determined with ΔCt method, where $\Delta Ct = Ct$ (miRNA of interest) $-$ Ct (reference miR-39).

Statistical analysis

The descriptive data was expressed as median (range) or mean $\pm$ SD. Median values of quantitative variables were used as cut-off for high and low values. The mean of 2 groups were compared using Student's t test. The Pearson correlation coefficient was measured for linear relationships between 2 continuous variables. Kaplan-Meier curve was obtained for survival analysis followed by log rang test to compare the statistical significance between the groups. Overall survival (OS) was defined as the length of time from the date of diagnosis to death or last follow-up. Progression-free survival (PFS) was defined as the length of time from date of diagnosis to date of progression, relapse or death. The end point of the study was March 7,

2019. For sensitivity and specificity of miRNA, receiver operating characteristic (ROC) curve was drawn and area under the curve (AUC) was calculated. All statistical analysis was done using STATA 11.2. $P < 0.05$ was considered as statistically significant.

Results

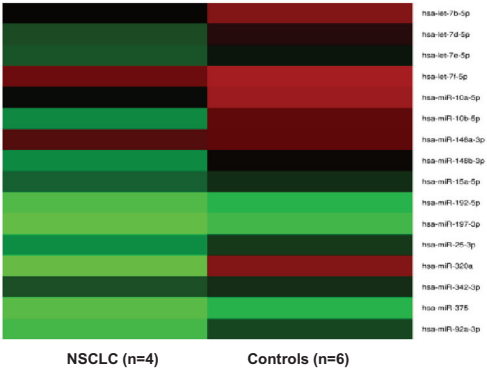
Demographic characteristics

The demographic characteristics of lung cancer patients as well as controls are summarized in Supplementary Table S1. The mean age of 75 NSCLC patients and 40 controls was 56.2 and 55.3 years, respectively ($P = 0.3242$). Majority of the NSCLC patients and controls were males (82.7% and 67.5%, respectively; $P = 0.054$). Histologically, there were 42 LUAD (31 males and 11 females) and 33 LUSC (31 males and 2 females) patients. The mean age of LUAD and LUSC patients was 53.6 years and 59.6 years, respectively. Almost two-third (67%) of all NSCLC patients were smoker with a median smoking index of 450 (range: 40-4000; Supplementary Table S1). Out of 42 LUAD, 21 (50%) were nonsmokers while almost 88% of all LUSC (29 out of 33) were smokers.

Identification of differentially expressed miRNAs using small RNA sequencing and their validation using qRT-PCR

Using small RNA sequencing, a total of 16 miRNAs were found to be differentially expressed in the serum of NSCLC patients (Fig 1). The mean reads per kilobase of transcript per million

A



B

MicroRNAs	Mean RPKM in NSCLC	Mean RPKM in Controls	Fold Change*
let-7b-5p	5779.5	60496.0	10.5
let-7d-5p	1510.6	7671.2	5.1
let-7e-5p	1007.3	4613.6	4.6
let-7f-5p	37814.0	101186.0	2.7
miR-10a-5p	5252.1	88384.0	16.8
miR-10b-5p	721.0	23992	33.3
miR-148a-3p	10604.0	22573.2	2.1
miR-148b-3p	708.1	6030.4	8.5
miR-15a-5p	941.7	2988.5	3.2
miR-192-5p	196.4	430.9	2.2
miR-197-3p	60.7	295.5	4.9
miR-25-3p	688.0	2410.7	3.5
miR-320a	45.0	61189	1358.6
miR-342-3p	1317.8	3089.6	2.3
miR-375	115.9	444.9	3.8
miR-92a-3p	273.6	1769.4	6.5

*$p < 0.05$

Fig. 1. Differential expression of circulating miRNAs in the serum of NSCLC as identified through small RNA sequencing on illumina HiSeq 2000 NGS platform. (A) Heat map of differentially expressed miRNAs (fold change >2.0; $P < 0.05$) in the serum of NSCLC as compared to controls. (B) The list of most significantly down-regulated miRNAs (fold change >2.0; $P< 0.05$) along with their reads per kilobase of transcript per million mapped reads (RPKM) values in NSCLC patients and controls.

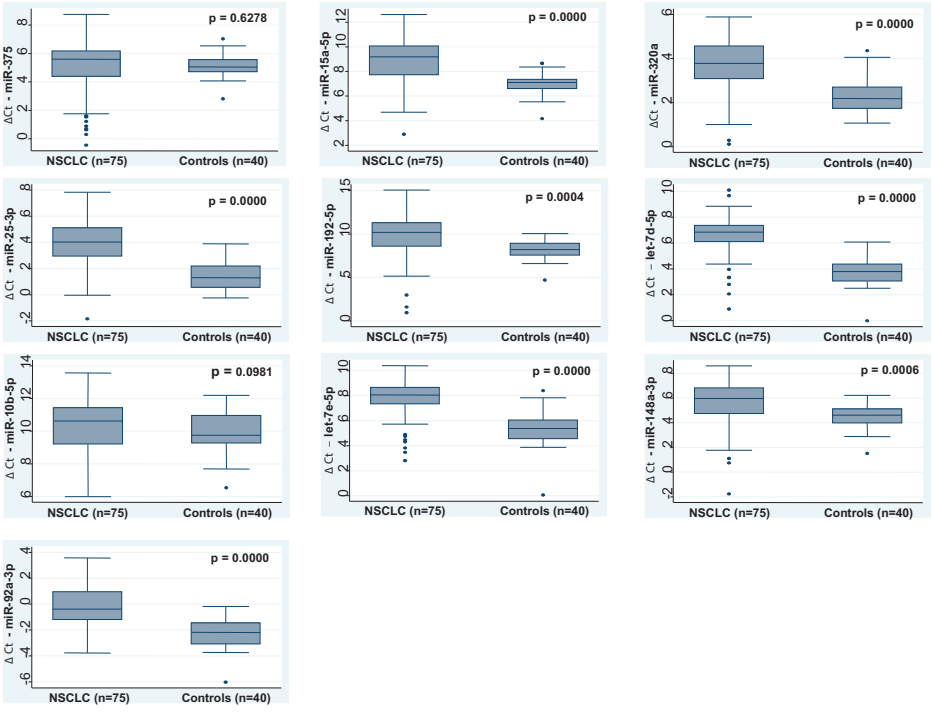


Fig. 2. Differential expression of circulating serum miRNAs in NSCLC patients as compared to controls. Box plot for the expression of miR-375, miR-320a, miR-15a-5p, miR-25-3p, miR-192-5p, let-7d-5p, miR-10b-5p, miR-92a-3p, miR-148a-3p, and let-7e-5p in NSCLC and controls.

mapped reads (RPKM) values of each miRNAs along with their fold change in expression for NSCLC patients as compared to controls is mentioned in Fig. 1B. We validated the expression of 10 differentially expressed miRNAs in the serum of 75 NSCLC patients and 40 controls using qRT-PCR. Except miR-375 and miR-10b-5p, all other miRNAs were significantly downregulated in the serum of NSCLC patients (Table 1; Fig 2). Interestingly, when we compared the expression of miR-375 and miR-10b-5p in LUAD, LUSC, and controls, we found a significant downregulation in their expression only in LUSC as compared to controls (Supplementary Table S2).

We assessed the diagnostic performance of all miRNAs using ROC curves (Table 1, Fig 3). Amongst all miRNAs, let-7d-5p performed the best with AUC of 0.917 and a sensitivity of 76% at 100% specificity (Table 1, Fig 3). Few miRNAs, such as miR-375 and miR-10b-5p, were found to be unsuitable as diagnostic biomarkers due to very low AUC and sensitivity (Table 1, Fig 3).

Correlation of miRNA expression with survival outcome and therapeutic response

The median OS and PFS of the NSCLC patient was 6.4 (range: 0.2-30.7) and 5.7 (range: 0.2-30.7) months, respectively. The NSCLC patients with higher expression of miR-15a-5p had better OS as compared to patients with lower expression of miR-15a-5p, however, the difference in OS did not achieve statistical significance ($P = 0.0789$; Table 2). To correlate miRNA expression with therapeutic response, patients who achieved complete remission, partial remission, or stable disease were classified as responders while patients with progressive diseases were classified as nonresponders. We found a lower expression of miR-320a in responders than in nonresponders (mean ΔCt of 3.7 vs 3.1, respectively; Table 3), although the data was not statistically significant ($P = 0.0784$).

Table 1
List of circulating miRNAs differentially expressed in the serum of NSCLC patients as compared to controls and their diagnostic performance.

MicroRNA	Mean ΔCt ± SE in NSCLC (n = 75)	Mean ΔCt±SE in controls (n = 40)	*P* value	log2FC*	AUC ± SE	95%CI	Cut-off (ΔCt values)	Sensitivity at 100% specificity
miR-375	5.01 ± 0.24	5.12 ± 0.12	0.6278	0.11	0.577 ± 0.053	0.478-0.666	≥7.165	12.0%
miR-15a-5p	8.89 ± 0.21	7.00 ± 0.13	**0.0000**	**−1.90**	0.833 ± 0.038	0.754-0.897	≥8.805	58.7%
miR-320a	3.73 ± 0.14	2.35 ± 0.13	**0.0000**	**−1.38**	0.844 ± 0.038	0.764-0.904	≥4.37	28.0%
miR-25-3p	3.9 ± 0.19	1.5 ± 0.18	**0.0000**	**−2.38**	0.882 ± 0.032	0.804-0.932	≥3.955	53.3%
miR-192-5p	9.67 ± 0.3	8.2 ± 0.17	**0.0004**	**−1.49**	0.772 ± 0.043	0.686-0.847	≥10.19	50.7%
let-7d-5p	6.44 ± 0.18	3.8 ± 0.18	**0.0000**	**−2.64**	0.917 ± 0.027	0.846-0.957	≥6.1	76.0%
miR-10b-5p	10.34 ± 0.18	9.98 ± 0.19	0.0981	−0.36	0.587 ± 0.055	0.487-0.674	≥12.43	6.67%
let-7e-5p	7.7 ± 0.17	5.35 ± 0.22	**0.0000**	**−2.35**	0.875 ± 0.034	0.804-0.932	≥8.435	33.3%
miR-148a-3p	5.55 ± 0.2	4.55 ± 0.14	**0.0006**	**−1.0**	0.751 ± 0.045	0.658-0.824	≥6.24	38.7%
miR-92a-3p	−0.18 ± 0.17	−2.23 ± 0.19	**0.0000**	**−2.05**	0.857 ± 0.035	0.784-0.918	≥-0.125	45.3%

95% CI, 95% confidence interval; AUC, area under the curve; NSCLC, non–small cell lung cancer; SE, standard error.
* log2 fold change (bold numbers indicate $P < 0.05$).

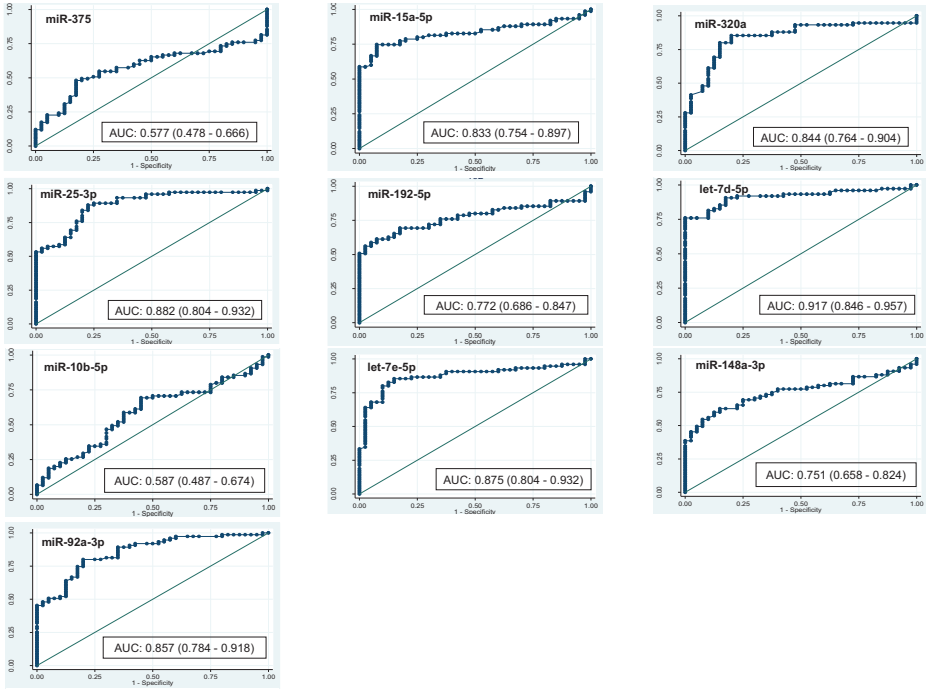


Fig. 3. Receiver operating characteristics (ROC) curve analysis for discriminating NSCLC using circulating serum miRNAs. The area under the ROC curve (AUC) along with 95% confidence interval (CI) is mentioned for all miRNAs in their individual figures.

Correlation of miRNA expression with clinicopathologic parameters

The median PFS was significantly higher for responders than nonresponders ($P = 0.0058$; Supplementary Fig S1.A, Supplementary Table S3). The PFS was also significantly correlated with age ($P = 0.0450$; Supplementary Fig S1.B, Supplementary Table S3). The median OS was higher for responders than nonresponders, although it was not statistically significant ($P = 0.0747$; Supplementary Fig S1.C, Supplementary Table S3).

A significant correlation was observed between the expression of miR-320a, miR-25-3p, and miR-148a-3p and disease stage (Supplementary Table S4). miR-375 expression was significantly correlated with lymph node involvement ($P = 0.0224$) and pleural effusion ($P = 0.0148$) while miR-10b-5p was also correlated with pleural effusion ($P = 0.0037$). Further, miR-320a, miR-25-3p, miR-192-5p, let-7d-5p, miR-148a-3p, and let-7e-5p were significantly correlated with gender of patients (Supplementary Table S4).

The expression of miR-25-3p was strongly correlated with miR-92a-3p ($r = 0.8678$; $P = 0.0000$) and miR-320a ($r = 0.8188$; $P = 0.0000$; Supplementary Table S5). The expression of miR-375 was positively correlated with miR-10b-5p while negatively correlated with miR-92a-3p (Supplementary Table S5). The expression of miR-15a-5p was found to be positively correlated with all miRNAs, except miR-375. All other correlations are being summarized in Supplementary Table S5.

Discussion

The presence of circulating tumor DNA, mRNA, and miRNA in the serum and plasma of cancer patients has sparked great interest because conventional diagnostic tests tend to be

Table 2
Correlation between circulating serum miRNA expression and survival in NSCLC.

miRNA	OS* ± SE (95%CI)	*P* value	PFS* ± SE (95%CI)	*P* value
miR-375				
<5.33	38.5 ± 11.3 (17.5-59.3)	0.8602	10.9 ± 9.2 (0.92-35.0)	0.8022
≥5.33	11.5 ± 9.9 (0.76-37.0)		14.0 ± 10.9 (1.4-40.4)	
miR-15a-5p				
<8.13	47.6 ± 15.2 (17.8-72.7)	**0.0789**	0	0.2930
≥8.13	15.0 ± 8.9 (3.0-35.9)		15.8 ± 8.2 (4.1-34.5)	
miR-320a				
<3.3	23.6 ± 12.4 (5.3-49.2)	0.2483	12.3 ± 10.2 (1.0-38.1)	0.3891
≥3.3	28.8 ± 10.8 (10.6-50.2)		15.6 ± 8.7 (3.6-35.6)	
miR-25-3p				
<3.1	30.4 ± 13.1 (8.9-55.6)	0.9815	10.4 ± 9.4 (0.7-35.5)	0.8255
≥3.1	23.3 ± 10.5 (6.9-45.2)		16.2 ± 8.8 (3.8-36.2)	
miR-192-5p				
<9.2	43.1 ± 15.3 (14.6-69.1)	0.3406	21.7 ± 12.2 (4.3-47.7)	0.5375
≥9.2	19.6 ± 9.1 (5.7-39.4)		9.9 ± 8.2 (0.9-31.6)	
let-7d-5p				
<6.1	31.7 ± 17.2 (5.5-63.4)	0.3823	16.9 ± 14.5 (1.0-50.1)	0.3302
≥6.1	24.1 ± 9.2 (9.0-43.1)		13.9 ± 7.6 (3.4-31.7)	
miR-10a-5p				
<10.5	22.7 ± 17.8 (1.5-59.1)	0.7058	25.5 ± 12.3 (6.5-50.5)	0.8153
≥10.5	23.6 ± 9.1 (8.7-42.5)		9.1 ± 7.6 (0.8-29.8)	
let-7e-5p				
<7.5	20.8 ± 16.6 (1.4-56.1)	0.6098	14.8 ± 12.8 (0.9-45.5)	0.6259
≥7.5	25.7 ± 9.6 (9.7-45.2)		13.6 ± 7.6 (3.1-31.6)	
miR-148a-3p				
<5.23	28.9 ± 12.5 (8.6-53.3)	0.4349	13.8 ± 11.2 (1.2-41.2)	0.4778
≥5.23	22.4 ± 11.7 (5.2-47.0)		14.0 ± 8.3 (2.9-33.7)	
miR-92a-3p				
<−0.96	41.7 ± 15.4 (13.6-68.1)	0.1249	17.8 ± 14.3 (1.3-50.0)	0.1153
≥−0.96	18.6 ± 8.8 (5.4-38.0)		11.9 ± 6.8 (2.7-28.5)	

Bold number indicates $P < 0.05$.
OS, overall survival; PFS, progression-free survival; SE, standard error.
* OS and PFS at 24 months.

imperfect and more invasive, posing logistic difficulties for serial tumor sampling. Detection of molecular alterations in circulating tumor DNA using droplet digital polymerase chain reaction (ddPCR) and next generation sequencing (NGS) has opened new avenues to clinicians for making treatment decisions based on the presence of specific targetable mutations. The stability of mRNA in circulation is a major issue limiting its utility as potential cancer biomarker. miRNAs are known to be more stable in the circulation as they are shielded from degradation by membrane vesicles and biopolymer complexes.[17] Hence, they are extensively being explored as cancer biomarkers.[8-12] However, so far, only 2 blood-based miRNA panels have successfully progressed to clinical testing as complementary tests for LDCT.[13,14] Hence, initial premise of this study was to understand the role of circulating serum miRNAs as diagnostic and prognostic biomarker as well as predictor of survival and therapeutic response in Indian NSCLC patients.

In this study, we identified 16 differentially expressed miRNAs in the serum of NSCLC patients (Fig 1). In validation cohort, we found significant downregulation of all miRNAs, except miR-375 and miR-10b-5p in NSCLC (Table 1; Fig 2). This suggests that suppression of the expression of specific miRNAs might contribute to disease progression, possibly by enhancing the expression of oncogenic molecules, and detection of these downregulated miRNAs may aid in differentiating early stage NSCLC patients from advanced stage patients. However, we could not validate this in the present study since our patient population had only stage III and IV patients. Few studies have used miRNAs for early detection of lung cancer. In a study, combination of a plasma-based miRNA signature classifier and LDCT resulted in a 5-fold reduction of LDCT false-positive rate to 3.7%.[14] Interestingly, plasma miRNAs could predict lung cancer even

Table 3
Correlation between the expression of circulating serum miRNA and therapeutic response in NSCLC.

MicroRNA	Mean ΔCt ± SE in responders* (n = 23)	Mean ΔCt ± SE in nonresponders† (n = 10)	*P* value
miR-375	5.03 ± 0.37 (4.3-5.8)	5.28 ± 0.79 (3.5-7.1)	0.6275
miR-15a-5p	8.3 ± 0.37 (7.5-9.1)	8.3 ± 0.65 (6.8-9.8)	0.5044
miR-320a	3.7 ± 0.21 (3.3-4.2)	3.1 ± 0.40 (2.2-4.0)	**0.0784**
miR-25-3p	3.6 ± 0.30 (2.9-4.2)	3.6 ± 0.37 (2.8-4.4)	0.5341
miR-192-5p	8.5 ± 0.64 (7.1-9.8	9.4 ± 0.78 (7.6-11.1)	0.7921
let-7d-5p	5.9 ± 0.33 (5.2-6.6)	6.2 ± 0.76 (4.5-7.9)	0.6674
miR-10b-5p	10.1 ± 0.27 (9.5-10.7)	10.4 ± 0.44 (9.4-11.4)	0.6961
let-7e-5p	7.2 ± 0.32 (6.6-7.9)	6.9 ± 0.65 (5.5-8.4)	0.3317
miR-148a-3p	4.9 ± 0.38 (4.2-5.7)	4.9 ± 0.79 (3.1-6.7)	0.4739
miR-92a-3p	−0.66 ± 0.27 (−1.2 to 1.0)	−0.57 ± 0.45 (−1.6 to 0.4)	0.5732

Bold number indicates $P < 0.05$.
SE, standard error.
* Responders: patients with CR + PR + SD.
† Nonresponders: patients with PD.

24 months ahead suggesting its potential of early detection.[14] Similarly, a panel of 6 plasma miRNAs discriminated lung cancer from asymptomatic high-risk subjects.[18] The expression of miR-17, miR-190b, and miR-375 were able to discriminate SCLC from NSCLC[18] suggesting that miRNAs may have potential for histologic classification. We also found that miR-375 and miR-10b-5p were significant downregulation in LUSC patients than controls (Supplementary Table S2). Although, miR-375 expression was higher in LUAD than LUSC, the difference did not reach statistical significance. This is in line with another study, where miR-375 levels were significantly higher is LUAD than LUSC[19] or normal lung tissue.[20] This suggests that downregulation of miR-375 and miR-10b-5p may have some role in LUSC, which is yet to be proven.

A number of studies have looked at the diagnostic potential of circulating miRNAs for lung cancer. Detection of 34 serum miRNAs identified early stage NSCLC patients with 80% accuracy.[21] miR-1254 and miR-574-5p discriminated early stage NSCLC patients from controls with a 73% sensitivity and 71% specificity.[22] A combination of miR-15b and miR-27b discriminated NSCLC from healthy controls with 100% sensitivity 84% specificity.[23] Another study identified a serum-based 4-miRNA signature that discriminated lung cancer patients from controls.[24] Similarly, 10 miRNA ratios comprising of 14 miRNAs discriminated lung cancer patients from controls with AUC of 0.979.[25] Montani et al[13] observed 74.9% overall accuracy, 77.8% sensitivity, and 74.8% specificity of miR-Test (a serum-based 13 miRNA signature) in 1115 high-risk individuals enrolled in the continuous observation of smoking Subjects lung cancer screening programme, Interestingly, amongst 13 miRNA signature of miR-Test, miR-92a-3p, miR-7d-5p, and miR-148a-3p are also reported in our study.

Consistent with other reports, our study revealed significant differences in miRNA expression between the cancer and cancer-free controls (Table 2). It is important to note that since only a limited number of miRNAs are truly tissue- or organ-specific, changes in the expression of majority of miRNAs found in our study may not reflect the lung cancer-specific miRNA expression patterns. We can presume that the variation in the expression of miRNAs might be due to disease condition since expression levels of serum miRNAs among healthy subjects were found to be quite consistent.[26] Further, most of the miRNAs mentioned in Table 1 have already been reported in the development of lung and other solid cancers.

The expression of miR-375 is known to be downregulated in tissue and plasma of NSCLC.[27-29] Recently, miR-375 was found to be over-expressed in SCLC than asymptomatic high-risk and NSCLC.[30] YAP1 and claudin-1 are the direct targets of miR-375.[31,32] MiR-15a and miR-16 cluster, which are known to regulate cell cycle and apoptosis, is frequently deleted in NSCLC.[33] The expression of miR-15a was significantly downregulated in NSCLC.[33-35] RB1 and BCL2L2 are the known targets of miR-15a.[33,34] The expression of miR-320a was found to be reduced in NSCLC tissue.[36-38] VDAC1, ELF4, IGF1R, STAT3, and SND1 are the direct targets of miR-320a in

NSCLC.[36-40] Ectopic overexpression of miR-320a blocked tumor cell proliferation and invasion, both in vitro and in vivo, by inhibiting VDAC1,[36] STAT3,[39] and PI3K/Akt[40] signaling.

Most of the available literature suggests that the expression of miR-25 is upregulated in lung cancer,[41] which is in contrast to our findings. However, literature does suggest the dual role of miR-25 in carcinogenesis. The possible oncogenic effect of miR-25 might be due to inhibition of FBXW7, RGS3, MOAP-1, KLF4, and BTG2 in NSCLC.[41,42] A number of studies have reported upregulation of miR-25 expression in NSCLC tissue and plasma.[42-44] In contrast, Zaporozhchenko et al[45] found downregulation of plasma miR-25 levels in lung cancer patients than controls. Interestingly, in 1 study, there was no significant difference in the expression of miR-25 in the plasma of NSCLC patients and healthy controls.[46] Few studies have reported the downregulation of miR-25 expression in ovarian cancer, prostate cancer, thyroid cancer, and colorectal cancer suggesting that miR-25 may also act as tumor suppressor.[41]

MiR-192-5p is known to have tumor suppressive functions in NSCLC since reduced expression of miR-192-5p was observed in NSCLC tissues.[47] Curcumin induced expression of miR-192-5p as well as ectopic expression of miR-192-5p in NSCLC cells suppressed cell proliferation and increased apoptosis.[48,49] Nicotine was found to promote NSCLC cell proliferation and epithelial-mesenchymal transition (EMT) by downregulating miR-192.[50] RB1 and XIAP are the targets of miR-192 in NSCLC.[47,49] let-7d and let-7e are 2 of the 13 known members of let7 family and are downregulated in many cancer types.[51,52] The expression of let-7d-5p was downregulated in the tumor-derived exosomes from the plasma of early stage LUAD patients.[53] Similarly, the reduced expression of let-7e-5p has been reported in the tumor-derived exosomes from the plasma,[53] serum,[54] and tumor tissue[54,55] of NSCLC patients. let-7 family members have >300 predicted target genes some of which are KRAS, MYC, IGF1R, TGFBR1, IGF2BP1, and HMGA2.[52]

We found a significant downregulation in the expression of miR-10b-5p in the serum of LUSC patients, but not in LUAD patients, when compared to controls (Supplementary Table S2). One recent study also found that the expression of miR-10b-5p was downregulated in the tumor-derived exosomes from the plasma of early stage LUSC patients.[53] Promoter DNA methylation might be one of the reason for downregulation in the expression of miR-10b.[56] MiR-148a-3p is another tumor suppressor miRNA, which is found downregulated in tumor tissue, serum, and plasma of NSCLC patients.[57-63] The ectopic expression of miR-148a inhibited cell proliferation and EMT by reducing the expression of SOS2, ROCK1, STAT3, and Wnt1.[58,59,63,64]

miR-92a belongs to miR-17-92 gene cluster, which functions as oncogene.[65] The expression of miR-92 was found to be upregulated in tumor tissue and plasma of NSCLC patients.[66-69] The overexpression of miR-92a promotes EMT by targeting phosphatase and tensin homologue (PTEN).[66,67] The induction of miR-92a expression by STAT3 is also known to promote lung cancer invasion by suppressing reversion-inducing-cysteine-rich protein with kazal motifs (RECK).[70] These reports suggest that miR-92a function like an OncomiR. Surprisingly, we observed downregulation of miR-92a-3p expression in the serum of NSCLC patients. There are other studies which also reported reduced expression of miR-92a in plasma samples of patients of multiple myeloma,[71] acute myeloid leukemia (AML),[72] and non-Hodgkin's lymphoma.[73] The level of miR-92a was significantly lower in both tumor tissues and serum samples of breast cancer patients than in that of healthy controls.[74] In patients with hepatocellular carcinoma, miR-92a has been reported to be overexpressed in the cancerous tissue whereas levels of miRNA in plasma were lower compared to healthy donors.[75]

We could not find any significant correlation between the expression of any of the 10 miRNAs with OS, PFS, and therapeutic response. There are studies which have shown miRNAs as good as well as poor prognostic factors in NSCLC. In one of the study, postoperative OS of lung cancer patients with lower miR-320a expression levels in tumor tissue was significantly shorter.[38] Similarly, underexpression of miR-375 in tumor tissue of NSCLC was an unfavorable prognostic factor for OS.[27,29] Reduction of let-7e expression in tumor tissue was strongly associated with shorter OS in NSCLC patients.[54,55] Low expression of miRNA-148a was also strongly associated with a higher risk of tumor-related death in NSCLC.[57,59] In a recent meta-analysis, a significant association was observed between low miR-148a level and poor OS in cancer pa-

tients.[76] In other studies, high plasma levels of miR-92 was found to be associated with worse OS and DFS,[67,68] while high expression levels of exosomal miR-10b-5p[77] and tissue miR-10b[78] was associated with poorer OS in NSCLC. A recent meta-analysis revealed that up-regulation of miR-10b could confer an unfavorable factor for OS.[79]

Like most of other studies, we do feel that our study has certain strengths and limitations. We used global miRNA profiling using NGS to identify differentially expressed miRNAs and validated the expression of 10 miRNAs in 115 serum samples using qRT-PCR. We not only tested the diagnostic performance of all miRNAs, but we also correlated their expression with OS, PFS, therapeutic response, and various clinicopathologic parameters. This is the first such comprehensive report on circulating miRNAs in Indian NSCLC population. However, our study has its own limitations, few of which are small sample size, shorter follow-up data, and absence of early stage NSCLC patients. It would have been interesting to analyze the expression of miRNAs in high-risk asymptomatic subjects and early stage and advanced stage NSCLC patients to find the utility of serum-based miRNAs in screening, risk assessment and early detection. Further, it would have been better to also profile the expression of studied miRNAs in tumor tissue since miRNAs from other tissues or organs which are released in the blood serum might mask the expression of lung cancer-specific miRNAs.

Conclusion

In summary, the expression of majority of miRNAs was downregulated in the serum of NSCLC patients as compared to controls. Few miRNAs, such as miR-375 and miR-10b-5p were differentially expressed in LUSC vs controls, but not between LUAD vs controls. Some of the miRNAs, such as miR-375 and miR-320a, are less studied for their involvement in the pathogenesis of NSCLC. Hence, further mechanistic studies are warranted to elucidate their role in disease biology and as candidate biomarkers for diagnosis and prognosis of NSCLC. Further, diagnostic and prognostic role of all these miRNAs should be validated in larger cohort of NSCLC patients with longer follow-up.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.currproblcancer.2020.100540.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394–424.
2. Ramalingam SS, Owonikoko TK, Khuri FR. Lung cancer: New biological insights and recent therapeutic advances. *CA Cancer J Clin*. 2011;61:91–112.
3. Pao W, Girard N. New driver mutations in non-small-cell lung cancer. *Lancet Oncol*. 2011;12:175–180.
4. Korpanty GJ, Graham DM, Vincent MD, Leighl NB. Biomarkers that currently affect clinical practices in lung cancer: EGFR, ALK, MET, ROS-1, and KRAS. *Front Oncol*. 2014;4:204.
5. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281–297.
6. Kasinski AL, Slack FJ. MicroRNAs en route to the clinic: Progress in validating and targeting microRNAs for cancer therapy. *Nat Rev Cancer*. 2011;11:849–864.
7. Singh DK, Bose S, Kumar S. Role of microRNA in regulating cell signaling pathways, cell cycle, and apoptosis in non-small cell lung cancer. *Curr Mol Med*. 2016;16:474–486.
8. Leidinger P, Keller A, Meese E. MicroRNAs—Important molecules in lung cancer research. *Front Genet*. 2012;2:104.
9. Jansson MD, Lund AH. MicroRNA and cancer. *Mol Oncol*. 2012;6:590–610.
10. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: Diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med*. 2012;4:143–159.
11. Rupaimoole R, Calin GA, Lopez-Berestein G, Sood AK. miRNA deregulation in cancer cells and the tumor microenvironment. *Cancer Discov*. 2016;6:235–246.
12. He Y, Lin J, Kong D, et al. Current state of circulating microRNAs as cancer biomarkers. *Clin Chem*. 2015;61:1138–1155.

13. Montani F, Marzi MJ, Dezi F, et al. miR-Test: A blood test for lung cancer early detection. *J Natl Cancer Inst*. 2015;107:djv063.
14. Sozzi G, Boeri M, Rossi M, et al. Clinical utility of a plasma-based miRNA signature classifier within computed tomography lung cancer screening: A correlative MILD trial study. *J Clin Oncol*. 2014;32:768–773.
15. Detterbeck FC, Boffa DJ, Kim AW, Tanoue LT. The eighth edition lung cancer stage classification. *Chest*. 2017;151:193–203.
16. Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). *Eur J Cancer*. 2009;45:228–247.
17. 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;8:467–477.
18. Lu S, Kong H, Hou Y, et al. Two plasma microRNA panels for diagnosis and subtype discrimination of lung cancer. *Lung Cancer*. 2018;123:44–51.
19. Patnaik S, Mallick R, Kannisto E, et al. MiR-205 and MiR-375 microRNA assays to distinguish squamous cell carcinoma from adenocarcinoma in lung cancer biopsies. *J Thorac Oncol*. 2015;10:446–453.
20. Gan TQ, Chen WJ, Qin H, et al. Clinical value and prospective pathway signaling of microRNA-375 in lung adenocarcinoma: A study based on the cancer genome atlas (TCGA), gene expression omnibus (GEO) and bioinformatics analysis. *Med Sci Monit*. 2017;23:2453–2464.
21. Bianchi F, Nicassio F, Marzi M, et al. A serum circulating miRNA diagnostic test to identify asymptomatic high-risk individuals with early stage lung cancer. *EMBO Mol Med*. 2011;3:495–503.
22. Foss KM, Sima C, Ugolini D, Neri M, Allen KE, Weiss GJ. miR-1254 and miR-574-5p—Serum-based microRNA biomarkers for early-stage non-small cell lung cancer. *J Thorac Oncol*. 2011;6:482–488.
23. Hennessey PT, Sanford T, Choudhary A, et al. Serum microRNA biomarkers for detection of non-small cell lung cancer. *PLoS ONE*. 2012;7:e32307.
24. Nadal E, Truini A, Nakata A, et al. A novel serum 4-microRNA signature for lung cancer detection. *Sci Rep*. 2015;5:12464.
25. Zaporozhchenko IA, Morozkin ES, Ponomaryova AA, et al. Profiling of 179 miRNA expression in blood plasma of lung cancer patients and cancer-free individuals. *Sci Rep*. 2018;8:6348.
26. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: A novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008;18:997–1006.
27. Li Y, Jiang Q, Xia N, Yang H, Hu C. Decreased expression of microRNA-375 in non-small cell lung cancer and its clinical significance. *J Int Med Res*. 2012;40:1662–1669.
28. Yu H, Jiang L, Sun C, et al. Decreased circulating miR-375: A potential biomarker for patients with non-small-cell lung cancer. *Gene*. 2014;534:60–65.
29. Chen LJ, Li XY, Zhao YQ, et al. Down-regulated microRNA-375 expression as a predictive biomarker in non-small cell lung cancer brain metastasis and its prognostic significance. *Pathol Res Pract*. 2017;213:882–888.
30. Lu S, Kong H, Hou Y, et al. Two plasma microRNA panels for diagnosis and subtype discrimination of lung cancer. *Lung Cancer*. 2018;123:44–51.
31. Nishikawa E, Osada H, Okazaki Y, et al. miR-375 is activated by ASH1 and inhibits YAP1 in a lineage-dependent manner in lung cancer. *Cancer Res*. 2011;71:6165–6173.
32. Yoda S, Soejima K, Hamamoto J, et al. Claudin-1 is a novel target of miR-375 in non-small-cell lung cancer. *Lung Cancer*. 2014;85:366–372.
33. Bandi N, Zbinden S, Gugger M, et al. miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer. *Cancer Res*. 2009;69:5553–5559.
34. Yang T, Thakur A, Chen T, et al. MicroRNA-15a induces cell apoptosis and inhibits metastasis by targeting BCL2L2 in non-small cell lung cancer. *Tumour Biol*. 2015;36:4357–4365.
35. Tafsiri E, Darbouy M, Shadmehr MB, Zagryazhskaya A, Alizadeh J, Karimipoor M. Expression of miRNAs in non-small-cell lung carcinomas and their association with clinicopathological features. *Tumour Biol*. 2015;36:1603–1612.
36. Zhang G, Jiang G, Wang C, et al. Decreased expression of microRNA-320a promotes proliferation and invasion of non-small cell lung cancer cells by increasing VDAC1 expression. *Oncotarget*. 2016;7:49470–49480.
37. Wang J, Shi C, Wang J, Cao L, Zhong L, Wang D. MicroRNA-320a is down-regulated in non-small cell lung cancer and suppresses tumor cell growth and invasion by directly targeting insulin-like growth factor 1 receptor. *Oncol Lett*. 2017;13:3247–3252.
38. Xing A, Pan L, Gao J. p100 functions as a metastasis activator and is targeted by tumor suppressing microRNA-320a in lung cancer. *Thorac Cancer*. 2018;9:152–158.
39. Lv Q, Hu JX, Li YJ, et al. MiR-320a effectively suppresses lung adenocarcinoma cell proliferation and metastasis by regulating STAT3 signals. *Cancer Biol Ther*. 2017;18:142–151.
40. Zhao W, Sun Q, Yu Z, et al. MiR-320a-3p/ELF3 axis regulates cell metastasis and invasion in non-small cell lung cancer via PI3K/Akt pathway. *Gene*. 2018;670:31–37.
41. Sarkozy M, Kahan Z, Csont T. A myriad of roles of miR-25 in health and disease. *Oncotarget*. 2018;9:21580–21612.
42. Ding X, Zhong T, Jiang L, Huang J, Xia Y, Hu R. miR-25 enhances cell migration and invasion in non-small-cell lung cancer cells via ERK signaling pathway by inhibiting KLF4. *Mol Med Rep*. 2018;17:7005–7016.
43. Yang T, Chen T, Li Y, et al. Down-regulation of miR-25 modulates non-small cell lung cancer cells by targeting CDC42. *Tumour Biol*. 2015;36:1903–1911.
44. Wang C, Ding M, Xia M, et al. A five-miRNA panel identified from a multicentric case-control study serves as a novel diagnostic tool for ethnically diverse non-small-cell Lung cancer patients. *EBioMedicine*. 2015;2:1377–1385.
45. Zaporozhchenko IA, Morozkin ES, Skvortsova TE, et al. Plasma miR-19b and miR-183 as potential biomarkers of lung cancer. *PLoS One*. 2016;11.
46. Zhang H, Mao F, Shen T, et al. Plasma miR-145, miR-20a, miR-21 and miR-223 as novel biomarkers for screening early-stage non-small cell lung cancer. *Oncol Lett*. 2017;13:669–676.

47. Feng S, Cong S, Zhang X, et al. MicroRNA-192 targeting retinoblastoma 1 inhibits cell proliferation and induces cell apoptosis in lung cancer cells. *Nucleic Acids Res*. 2011;39:6669–6678.
48. Jin H, Qiao F, Wang Y, Xu Y, Shang Y. Curcumin inhibits cell proliferation and induces apoptosis of human non-small cell lung cancer cells through the upregulation of miR-192-5p and suppression of PI3K/Akt signaling pathway. *Oncol Rep*. 2015;34:2782–2789.
49. Ye M, Zhang J, Zhang J, Miao Q, Yao L, Zhang J. Curcumin promotes apoptosis by activating the p53-miR-192-5p/215-XIAP pathway in non-small cell lung cancer. *Cancer Lett*. 2015;357:196–205.
50. Du X, Qi F, Lu S, Li Y, Han W. Nicotine upregulates FGFR3 and RB1 expression and promotes non-small cell lung cancer cell proliferation and epithelial-to-mesenchymal transition via down-regulation of miR-99b and miR-192. *Biomed Pharmacother*. 2018;101:656–662.
51. Boyerinas B, Park SM, Hau A, Murmann AE, Peter ME. The role of let-7 in cell differentiation and cancer. *Endocr Relat Cancer*. 2010;17:F19–F36.
52. Kolenda T, Przybyła W, Teresiak A, Mackiewicz A, Lamperska KM. The mystery of let-7d—A small RNA with great power. *Contemp Oncol (Pozn)*. 2014;18:293–301.
53. Jin X, Chen Y, Chen H, et al. Evaluation of tumor-derived exosomal miRNA as potential diagnostic biomarkers for early-stage non-small cell lung cancer using next-generation sequencing. *Clin Cancer Res*. 2017;23:5311–5319.
54. Zhu WY, Luo B, An JY, et al. Differential expression of miR-125a-5p and let-7e predicts the progression and prognosis of non-small cell lung cancer. *Cancer Invest*. 2014;32:394–401.
55. Zhang YK, Zhu WY, He JY, et al. miRNAs expression profiling to distinguish lung squamous-cell carcinoma from adenocarcinoma subtypes. *J Cancer Res Clin Oncol*. 2012;138:1641–1650.
56. Heller G, Altenberger C, Steiner I, et al. DNA methylation of microRNA-coding genes in non-small-cell lung cancer patients. *J Pathol*. 2018;245:387–398.
57. Chen Y, Min L, Zhang X, et al. Decreased miRNA-148a is associated with lymph node metastasis and poor clinical outcomes and functions as a suppressor of tumor metastasis in non-small cell lung cancer. *Oncol Rep*. 2013;30:1832–1840.
58. Li J, Song Y, Wang Y, Luo J, Yu W. MicroRNA-148a suppresses epithelial-to-mesenchymal transition by targeting ROCK1 in non-small cell lung cancer cells. *Mol Cell Biochem*. 2013;380:277–282.
59. Chen Y, Min L, Ren C, et al. miRNA-148a serves as a prognostic factor and suppresses migration and invasion through Wnt 1 in non-small cell lung cancer. *PLoS One*. 2017;12.
60. Li L, Chen YY, Li SQ, Huang C, Qin YZ. Expression of miR-148/152 family as potential biomarkers in non-small-cell lung cancer. *Med Sci Monit*. 2015;21:1155–1161.
61. Yang JS, Li BJ, Lu HW, et al. Serum miR-152, miR-148a, miR-148b, and miR-21 as novel biomarkers in non-small cell lung cancer screening. *Tumour Biol*. 2015;36:3035–3042.
62. Huang MX. Down-expression of circulating micro ribonucleic acid (miRNA)-148/152 family in plasma samples of non-small cell lung cancer patients. *J Cancer Res Ther*. 2016;12:671–675.
63. He M, Xue Y. MicroRNA-148a suppresses proliferation and invasion potential of non-small cell lung carcinomas via regulation of STAT3. *Onco Targets Ther*. 2017;10:1353–1361.
64. Xie Q, Yu Z, Lu Y, Fan J, Ni Y, Ma L. microRNA-148a-3p inhibited the proliferation and epithelial-mesenchymal transition progression of non-small-cell lung cancer via modulating Ras/MAPK/Erk signaling. *J Cell Physiol*. 2018. doi:10.1002/jcp.27899.
65. Zhang X, Li Y, Qi P, Ma Z. Biology of MiR-17-92 cluster and its progress in lung cancer. *Int J Med Sci*. 2018;15:1443–1448.
66. Ren P, Gong F, Zhang Y, Jiang J, Zhang H. MicroRNA-92a promotes growth, metastasis, and chemoresistance in non-small cell lung cancer cells by targeting PTEN. *Tumour Biol*. 2016;37:3215–3225.
67. Lu C, Shan Z, Hong J, Yang L. MicroRNA-92a promotes epithelial-mesenchymal transition through activation of PTEN/PI3K/AKT signaling pathway in non-small cell lung cancer metastasis. *Int J Oncol*. 2017;51:235–244.
68. Xu X, Zhu S, Tao Z, Ye S. High circulating miR-18a, miR-20a, and miR-92a expression correlates with poor prognosis in patients with non-small cell lung cancer. *Cancer Med*. 2018;7:21–31.
69. Zhu Q, Zang Q, Jiang ZM. Enhanced expression of non-coding miR-92a expression is implicated in the development of lung cancer. *Eur Rev Med Pharmacol Sci*. 2018;22:1028–1034.
70. Lin HY, Chiang CH, Hung WC. STAT3 upregulates miR-92a to inhibit RECK expression and to promote invasiveness of lung cancer cells. *Br J Cancer*. 2013;109:731–738.
71. Yoshizawa S, Ohyashiki JH, Ohyashiki M, et al. Down-regulated plasma miR-92a levels have clinical impact on multiple myeloma and related disorders. *Blood Cancer J*. 2012;2:e53.
72. Tanaka M, Oikawa K, Takanashi M, et al. Down-regulation of miR-92 in human plasma is a novel marker for acute leukemia patients. *PLoS One*. 2009;4:e5532.
73. Ohyashiki K, Umezu T, Yoshizawa S, et al. Clinical impact of down-regulated plasma miR-92a levels in non-Hodgkin's lymphoma. *PLoS One*. 2011;6:e16408.
74. Si H, Sun X, Chen Y, et al. Circulating microRNA-92a and microRNA-21 as novel minimally invasive biomarkers for primary breast cancer. *J Cancer Res Clin Oncol*. 2013;139:223–229.
75. Shigoka M, Tsuchida A, Matsudo T, et al. Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development. *Pathol Int*. 2010;60:351–357.
76. Duan F, Liu W, Fu X, et al. Evaluating the prognostic value of miR-148/152 family in cancers: based on a systemic review of observational studies. *Oncotarget*. 2017;8:77999–78010.
77. Liu Q, Yu Z, Yuan S, et al. Circulating exosomal microRNAs as prognostic biomarkers for non-small-cell lung cancer. *Oncotarget*. 2017;8:13048–13058.
78. Zhang J, Xu L, Yang Z, et al. MicroRNA-10b indicates a poor prognosis of non-small cell lung cancer and targets E-cadherin. *Clin Transl Oncol*. 2015;17:209–214.
79. Huang Q, Song Q, Zhong W, Chen Y, Liang L. MicroRNA-10b and the clinical outcomes of various cancers: A systematic review and meta-analysis. *Clin Chim Acta*. 2017;474:14–22.