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Potential circulating miRNA signature for early detection of NSCLC

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Circulating microRNAs (c-miRNAs) are promising biomarkers for screening, early detection and prognosis of cancer. The purpose of this investigation was to identify a panel of c-miRNAs in plasma that could contribute to early detection of non-small cell lung cancer (NSCLC). We profiled the expression of 44 unique plasma miRNAs in training set of 34 NSCLC patients and 20 matched healthy individuals by miRCURY LNA™ Universal RT microRNA PCR Panel and calculated dysregulation fold changes using the 2-ΔΔCt equation. Selected plasma miRNAs were then validated by SYBR green q-RT PCR using an independent validation set of plasma samples from NSCLC patients (n: 72) and NC (n: 50). In the validation set, the receiver operating characteristic (ROC) curves were generated for four miRNAs. In the training set, 17 miRNAs were significantly up-regulated and nine were down-regulated in the plasma from NSCLC patients versus matched normal controls. Four miRNAs (miR-21, miR-328, miR-375 and miR-141) were selected for validating their diagnostic value in the testing set. ROC plot analysis showed that a high specificity (98%) and sensitivity (82.7%) in miR-141 in comparing early NSCLC patient and controls. So among these four plasma miRNAs only miR-141 could be promising biomarkers for early detection of NSCLC. In addition to, we found a significant positive correlation between stage and miR-21 expression level (95% CI: 0.687-0.863; p-value < 0.0001). Considering the accessibility and stability of circulating miRNAs, plasma miR-141 is a useful biomarker early detection of NSCLC as a supplement in future screening studies.

Keywords Non-small cell lung cancer, microRNAs, plasma, non-invasive, early detection

Introduction

Lung cancer is the most common cause of cancer mortality in both men and women worldwide. Nearly 1.4 million deaths per year happen by lung cancer all around the world (1). Further, the mortality burden of lung cancer among females in developing countries is as high as the burden for cervical cancer, with each accounting for 11% of the total female cancer deaths (2,3). Lung cancer has two major pathological groups: 15% small cell lung cancer (SCLC) and 85% non-small cell lung cancer (NSCLC) (4). Squamous cell carcinoma (SCC), adenocarcinoma (ADC), adenosquamous cell carcinoma (ASC) and large cell carcinoma (LCC) are various subtypes of NSCLC (5).

Chest X-ray, *low-dose computed tomography* (LDCT), breath test and sputum cytology are considered for early detection of lung cancer (6,7). Additionally, several serum-based protein biomarkers have been recognized for this purpose, such as CA-125, CA19-9, carcinoembryonic antigen (CEA), CYFRA21-1, Chromogranin A, *neuron-specific enolase* (NSE) and tissue polypeptide-specific antigen (TPS) (7–9). However, they are non-invasive and cost-effective diagnostic

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methods, their sensitivity and specificity are limited (10). Therefore, there is considerable need to identify novel non-invasive biomarkers with superior accuracy for the screening of early-stage NSCLC.

MicroRNAs (miRNAs) are 19–25 nt small non-coding RNAs that negatively regulate the level of gene expression of ~30% protein coding genes by base pairing on 3' untranslated region (3' UTR), leading to mRNA degradation or translation inhibition (11). It is reported that many genes that are involved in basic biological functions such as cellular proliferation, differentiation, and apoptosis can be regulated by miRNAs (12–14).

MiRNAs can potentially regulate many key oncogenes or tumor suppressors, therefore their dysregulation could be an important mechanism of tumorigenesis (15,16) and in several types of tumors such as lung cancer (17) with dysregulation in their expression level (18).

MiRNAs have been recognized in different body fluids such as blood, urine, malignant ascites, Broncho alveolar lavage fluid, synovial fluid, breast milk and saliva that are called circulating miRNAs (c-miRNAs). Some actions like tissue damage, apoptosis, metastasis or inflammation can lead to energy-free passive miRNAs release from malignant tissues. On the other hand, miRNA can be secreted actively using ATP, similar to the release of hormones and cytokines into the circulation. As a rule, energy-free passive miRNA release does not play a major role in the generation of c-miRNAs despite of active and selective secretion (19). Thus, understanding the role of various miRNAs at different stages of lung cancer, along with their expression alteration, may suggest c-miRNAs as potential biomarkers for diagnosis and prognosis (20). Meanwhile, c-miRNAs as an early detection biomarker for lung cancer have much specificity (21).

In the present study, we have analyzed the profile of c-miRNA expression in plasma of NSCLC patients in comparison with normal plasma samples by quantitative real-time PCR to identify their potential utility for early detection and diagnosis of NSCLC.

Materials and methods

Plasma samples

We obtained blood samples from 72 NSCLC patients (including 41 adenocarcinoma and 31 squamous cell carcinoma) from 2013 to 2016, at Masih Daneshvari Hospital, Iran and 50 healthy matched volunteers were used as controls. All cases were staged according to the revised AJCC/UICC 7th edition TNM classification schema (22). The age, sex and smoking habit of healthy controls were matched with NSCLC patients. The study was approved by the ethics committee (sbmul. REC1394. 112) of the Masih Daneshvari Hospital and patients' written informed consent were obtained for all biological samples. The blood samples were collected in EDTA tubes at the time of initial consultation before surgical management and/or adjuvant therapy. Plasma was separated from the samples within 2 hours of collection by centrifugation. All processes of plasma extracting were done based on platelet poor plasma protocol (23). Plasma samples were transferred to fresh tubes and stored at –80 ˚C until further investigation.

Demographic and clinicopathological characteristics of samples

We have managed to do this research in two phases. The 34 NSCLC patients (men 62%) with age 60.5 ± 9.5 years were included in the training set. These samples were pooled in eight groups: A; six patients with ADC at stage I, B; four patients with ADC in stage II, C; four patients with ADC at stage IIIa, D; five patients with ADC at stage IIIb & IV, E; three patients with SCC at stage I, F; three patients with SCC at stage II, G; four patients with SCC at stage IIIa and H; five patients with SCC at an advanced stage (IIIb & IV). The 20 matched healthy subjects with age 59 ± 5.7 years were used in two pooled samples.

For validation set, 72 NSCLC patients (men 70%) with age 61.7 ± 10.1 years were analyzed from which, 72.2% patients were at early stages (stage I-IIIa) and 57% were suffered from adenocarcinoma. In parallel 50 matched control group (men 60%) with age 57.8 ± 8.1 years were included in the study (Table 1).

Training set: miRNA screening with miRCURY LNA™ universal RT microRNA PCR panel

RNA isolation and cDNA synthesis

From 100 µl plasma, total RNA was isolated by Trizol) Roche cat#11667165001) protocol (24) and First strand cDNA synthesis was performed using Universal cDNA synthesis kit II (EXIQON Co. cat#203301) according to manufacturer's instruction.

Accuracy and efficiency identification of isolated RNA and cDNA by RNA spike-ins

It should be noted that UniSp5 and cel-mir-39-3p spike-ins, which have the lowest concentration of all the RNA spike-in, were used to detect the accuracy and efficiency of RNA and cDNA. Therefore, the UniSp5 and cel-mir-39-3p spike-ins primer sets were purchased from EXIQON (Cat #203955 and #203952) and according to the specified ct, that determined by EXIQON, for these spike-ins all the cDNA samples for training set and testing set were analyzed through qRT-PCR. The SYBR Green RT-PCR reactions were performed in MicroAmp Optical 96-well plate using StepOne v.2.2.2 software (Applied Biosystems, USA) according to manufacturer's instruction. The thermal-cycling conditions were as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 sec and 60 °C for 10 sec.

qRT-PCR

In training set, eight pooled NSCLC RNA samples (including 24 early-stage and 10 advanced-stage) and two pooled control RNA (including 20 matched healthy samples) (Table 1) were examined for 44 miRNAs on miRCURY LNA™ Universal RT microRNA PCR Panel (EXIQON co. Cat#203893) by ExiLENT SYBR® Green master mix (EXIQON Co. Cat#203403). The miRNAs primer sets that were coated on panels were selected based on previous review and original articles (25–28). The SYBR Green RT-PCR reactions were performed according to manufacturer's instruction. Non-Template Control (NTC), inter plate calibrator (IPC) were included in each assay. The thermal-cycling conditions were as follows: 95 °C for 10 min,

Table 1 Demographic and clinical features of NSCLC patients

	Training Set		Testing Set	
	Control	NSCLC	Control	NSCLC
	N = 20	N = 34	N = 50	N = 72
Age				
Mean ± SD (year)	59 ± 5.7	60.5 ± 9.5	57.8 ± 8.1	61.76 ± 10.1
Gender				
Male	13(65%)	21(62%)	30(60%)	51(70%)
Female	7(35%)	13(38%)	20(40%)	21(30%)
Smoking history				
Non-smoker	7(35%)	11(32.4%)	18(36%)	19(26.4%)
Current smoker	13(65%)	23(67.6%)	32(64%)	53(73.6%)
Tumor subtype				
Adenocarcinoma	-	19(56%)	-	41(57%)
Squamouscell carcinoma	-	15(44%)	-	31(43%)
Tumor staging(TNM)				
Early				
I	-	9(26.5%)	-	9(12.5%)
II	-	7(20.5%)	-	26(36.1%)
IIIA	-	8(23.5%)	-	17(23.6%)
Advanced				
IIIB , IV	-	10(29.5%)	-	20(27.8%)

followed by 45 cycles of 95 °C for 10 sec and 60 °C for 10 sec.

Testing set: miRNA validation assay

RNA isolation and validation assay by qRT-PCR

According to the training set results miR-21, miR-328-3p, miR-375, miR-141 were selected for validation assay in 72 cases (including 41 ADC and 31 SCC) and 50 matched controls (Table 1). RNA extraction was performed by MiRNeasy Serum/Plasma kit (QIAGEN Co. Cat#217184) according to manufacturer's instruction. cDNA synthesis was performed by Universal cDNA synthesis kit II like training set plan and qRT-PCR run with ExiLENT SYBR® Green master mix. The qRT-PCR reactions were similar to spike-ins thermal-cycling condition. Then, the comparative ΔCt method was calculated for each miRNA. Finally, for designing expression level graphs $\log_2 2^{-\Delta\Delta Ct}$ of patients' plasma was used. The values less than 0.05 were indicated statistically significant.

Statistical analysis

NormFinder v20 (29) and geNorm V3.5 (30) softwares were used for choosing the reference gene in the training set. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ equation and miRNAs that had equal or more than 2-fold change in their expression level were selected.

All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA was used to test for associations between miRNAs expression level and clinicopathological features of the patients in testing set. Receiver operating characteristic (ROC) curve was used to interpret the ability of miRNAs in discriminating patients from healthy controls. The area under the curve (AUC), sensitivity and specificity were calculated. Both generation and analysis of the ROC curve were performed by MedCalc statistical software package 11.0.1 (MedCalc Software, bvba, Ostend, Belgium). P-values equal or lesser than 0.05 were considered statistically significant .

Results

miRNA expression profile in training set

To investigate the expression profile of miRNAs in the plasma of NSCLC patients, the panel was tested 44 for finding the potential deregulation in categorized pooled NSCLC plasma samples versus matched healthy samples. According to NormFinder v20 and geNorm V3.5 software we chose miR-24-3p (EXIQON Co. Cat#204260) as the most stable housekeeping miRNA among 44 miRNAs in the panel.

It is apparent from Figure 1 that 17 miRNAs had significant up-regulation (equal or more than 2-fold change) in expression level and 9 miRNAs had significant down-regulation.

The correlation between miRNA expression level and clinicopathological features

In this study, the expression level of miRNAs in patients in early- stage versus advance was analyzed. The data showed that eight miRNAs had a remarkable up-regulation and seven miRNA were down-regulated significantly in both early and advanced stages (Table 2).

The study of expression profile in ADC and SCC samples showed that 12 miRNAs had a significant up-regulation and eight miRNAs were down-regulated significantly in both ADC and SCC samples in comparison to normal samples. Interestingly, we found that miR-182 and Let-7a were down-regulated in ADC while they had a significant up-regulation in SCC samples. Moreover, miR-128-3p and miR-93 were

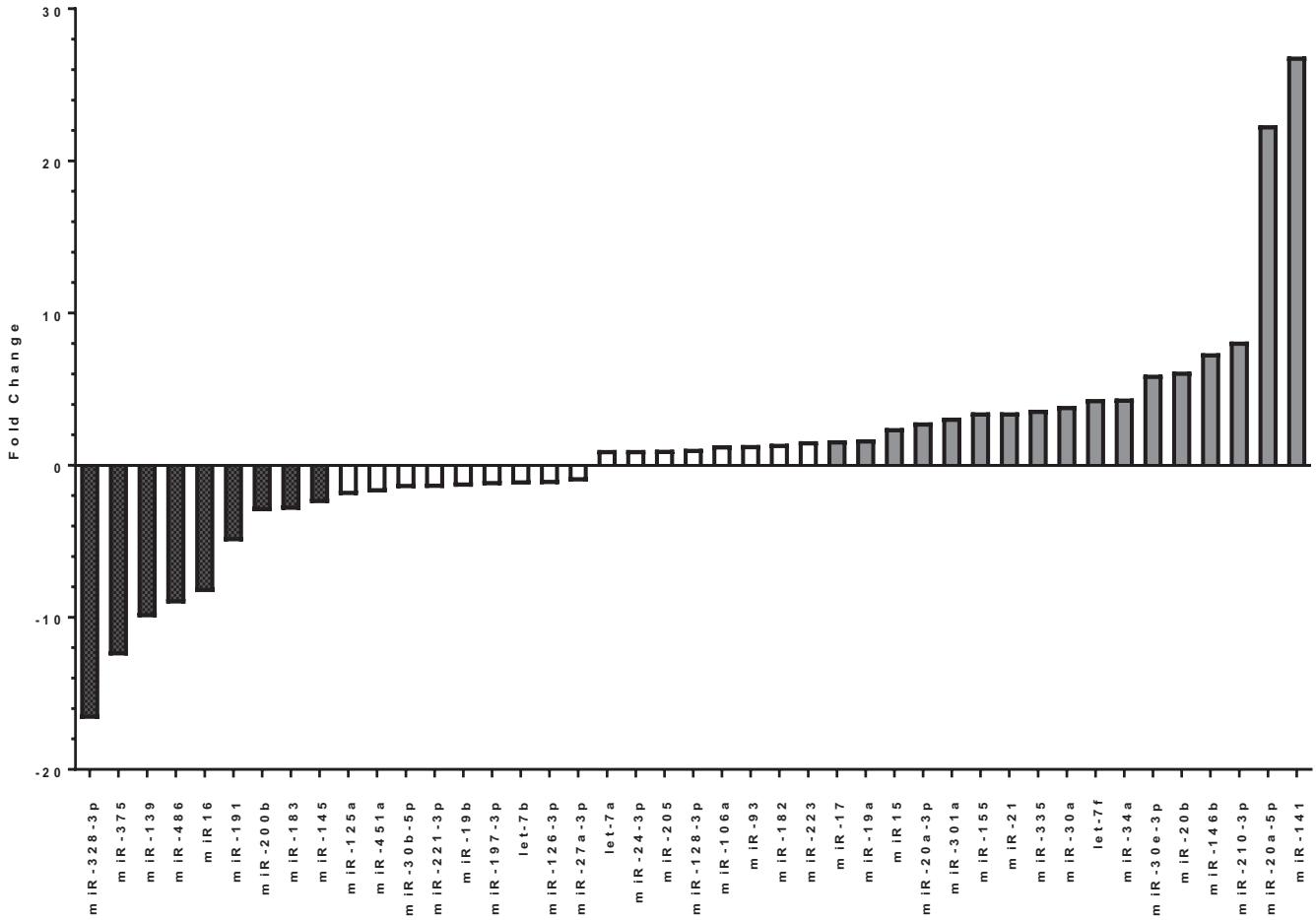

Figure 1 The expression level of 44 miRNAs in training set. Black columns show miRNAs had down-regulation, gray columns show miRNAs had up-regulation, and white columns do not show significant change in the level of expression.

up-regulated in ADC since they had a significant down-regulation in SCC. MiR-301-3p and miR-17 were up-regulated and miR-19b and miR-126-3p had a significant down-regulation only in SCC samples and they had no change in ADC samples. Let-7b, miR-15a and miR-19a were up-regulated and miR-27a-3p, miR-125a, miR-183 and miR-200b had a significant down-regulation only in ADC samples and they had no change in SCC samples. We did not find any correlation between miRNA expression level with sex, age and smoking in training test (Table 2).

miRNA expression validation in testing set

As it was mentioned, we selected four miRNAs (miR-21, miR-328-3p, miR-375 and miR-141) for study by qRT-PCR in 72 NSCLC plasma samples versus 50 matched healthy controls. The data showed that miR-141 expression level was significantly up-regulated in NSCLC samples versus matched normal controls (p-value < 0.0001). Importantly, miR-141 was up-regulated more significantly in early stage samples compared to advanced stage patients (p-value < 0.0001). It is noteworthy that miR-21 was increased significantly in advanced stage patients against matched controls (95% CI: 0.962-1.0; p-value < 0.0001). In addition, a significant positive correlation between stage and miR-21 expression level (95% CI: 0.687-0.863; p-value < 0.0001) was found. Our investigation showed that miR-328-3p expression level was down-regulated significantly in advanced stage patients versus matched healthy controls (95% CI: 0.949-1.0; p-value <0.0001). MiR-375 did not demonstrate any correlation between its expression levels with clinicopthological features (Table 3). ROC curve analysis was performed to identify the diagnostic value of four selected miRNAs. The results showed miR-141 had the highest area under curve (AUC: 0.918; 95% CI 0.949-1.0, p-value <0.0001) and the sensitivity and specificity were 82.7% and 98%, respectively (Figure 2, Table 3). MiR-375 had the lowest area under curve (AUC: 0.60; 95% CI 0.40-0.646, p-value <0.62) with 53.85% sensitivity and 74 % specificity.

Finally, we analyzed these miRNAs in combinations to find a unique panel from them. ROC curve, AUC and value of each combination are obtained (Table 4). MicroRNAs combination analysis among early NSCLC patients and controls clearly demonstrated significant correlation in all groups that including miR-141.

Discussion

Many investigations have reported that c-miRNAs are potentially non-invasive helpful diagnostic biomarkers for early

Table 2 The correlation between 44 miRNA expression level and clinicopathological characteristics of samples in NSCLC in training set.

Pathological Subtype	Early-stage (n: 34)			
Advanced-stage (n: 20)	**miRNA expression**	**Up-regulated**	**No change**	**Down-regulated**
	Up-regulated	Let-7f, miR-15a miR-19a, miR-20a-5p **miR-21,** miR-30e-3p miR-34a, **miR-141**	miR-93	miR-200b miR-451a
	No change	miR-20a-3p, miR-20b miR-146b, miR-155 miR-210-3p, miR-335 miR-301a-3p	Let-7a, Let-7b miR-17, miR-19b miR-30-5p, miR-197 miR-106a miR-126-3p, miR-182 miR-205, miR-221-3p	miR-16
	Down-regulated	miR-30a miR-128-3p miR-223	miR-27a-3p miR-125a	miR-139, miR-145 miR-183, mir-191 **miR-328-3p, miR-375** miR-486
Pathological Subtype	ADC (n:41)			
SCC (n:31)	**miRNA expression**	**Up-regulated**	**No change**	**Down-regulated**
	Up-regulated	Let-7f, miR-20a-3p miR-20a-5p, miR-20b **miR-21,** miR-30a miR-30e-3p, miR-34a **miR-141,** miR-146b miR-210-3p, miR-335	miR-17 miR-301-3p	Let-7a miR-182
	No change	Let-7b miR-15a miR-19a	miR-30b-3p, miR-106a miR-205, miR-223	miR-27a-3p, miR-125a miR-183, miR-200b
	Down-regulated	miR-93 miR-128-3p	miR-19b miR-126-3p	miR-16, miR-139 miR-145, miR-191 miR-197-3p, miR-486 **miR-328-3p, miR-375**

Four selected miRNAs for validating in testing set were shown in bold

detection of various cancers. MiRNAs are released into the blood circulation in energy-free passive or ATP-dependent active ways (31). MiRNAs in plasma could remain stable at room temperature for 24 hours and eight freeze-thaw cycles; while, synthetic miRNAs were rapidly degraded in plasma (32,33). The cause of this stability should be relevant to pack to microvesicles (such as exosomes and apoptotic bodies) or ribonucleoprotein complexes (such as argonaute 2). In addition, Pigati et al. reported that cells have a mechanism in place to select some miRNAs for cellular release or retention (34). So it is an important issue to choose a c-miRNA as a biomarker for different purposes.

In this study, the plasma miRNAs from 34 NSCLC patients and 20 NC subjects were profiled using miRCURY LNA™ Universal RT microRNA PCR Panel.

In this study, miR-1228 was selected as a reference miRNA based on previous study (35). In our experiment, this miRNA had undetermined ct in all panels, probably due to low amount. Therefore, miR-24-3p was selected by NormFinder and geNorm as reference gene.

We found 26 miRNAs significantly deregulated (17 up- and 9 down-regulated) in the NSCLC plasma versus NC. Then, we validated four miRNAs (miR-21, miR-141, and miR-328 and miR-375) by qRT-PCR. We observed maximum area under curve 0.918 with high specificity (82.7%) and high sensitivity (98.00%) for plasma miR-141 in discrimination of early NSCLC patients and normal controls. miR-141 was up-regulated in the patient plasma samples independent of age, sex, tumor pathology and stage. Although it has been reported for some cancers (33,36), to our best knowledge this is the first study that reports up-regulation of miR-141 in plasma of NSCLC patients.

We found a significant positive correlation between stage and miR-21 expression level (95% CI: 0.687-0.863; p-value < 0.0001). Our investigation showed that miR-328-3p expression level was down-regulated significantly just in advanced-stage patients versus matched healthy controls (95% CI: 0.949-1.0; p-value <0.0001). MiR-375 did not demonstrate any correlation between its expression levels with clinicopthological features.

MiR-141 is one of the well-known members of the miR-200 family, which has been reported to be associated with various cancers. It is down-regulated and acts as a potential tumor suppressor in gastric cancer, pancreatic ductal adenocarcinoma, pancreatic cancer, osteosarcoma, prostate cancer, hepatocellular, primary peritoneal carcinoma, choriocarcinoma, esophageal cancer, breast cancer and renal cell carcinoma (37). However, miR-141 has also

Table 3 Statistical Analysis and validation of four miRNAs for lung cancer diagnosis.

miRNAs	Groups		AUC	95% CI	P-Value	%Sensitivity	%Specificity
miR-21-5p	**Stage**	**Control: NSCLC**	0.616	0.492-0.730	0.0811	61.11	89.7
		Control : Early	**0.663**	**0.547-0.739**	**0.0101**	**55.77**	**100.00**
		Control : Advanced	**1.000**	**0.962-1.000**	**<0.0001**	**94.00**	**93.90**
		Early : Advanced	**0.784**	**0.687-0.863**	**<0.0001**	**94.80**	**78.43**
	Sub -Type	**Control : ADC**	0.625	0.511-0.730	0.1506	62.50	88.00
		Control : SCC	0.619	0.496-0.732	0.2729	61.90	91.10
		ADC : SCC	0.521	0.379-0.660	0.8136	57.14	62.50
miR-141	**Stage**	**Control: NSCLC**	**0.972**	**0.919-0.995**	**<0.0001**	**96.30**	**99.30**
		Control : Early	**0.918**	**0.949-1.000**	**<0.0001**	**82.69**	**98.00**
		Control : Advanced	**0.750**	**0.634-0.845**	**<0.0001**	**50.00**	**95.65**
		Early : Advanced	**0.750**	**0.634-0.845**	**<0.0001**	**89.90**	**91.83**
	Sub -Type	**Control : ADC**	**0.969**	**0.904-0.995**	**<0.0001**	**96.87**	**94.00**
		Control : SCC	**0.976**	**0.909-0.998**	**<0.0001**	**95.24**	**97.00**
		ADC : SCC	0.568	0.415-0.694	0.2618	32.69	98.00
miR-328-3p	**Stage**	**Control: NSCLC**	0.519	0.417-0.619	0.7873	51.85	79.00
		Control : Early	0.529	0.429-0.629	0.6769	52.94	98.00
		Control : Advanced	**1.000**	**0.949-1.000**	**<0.0001**	**89.00**	**91.09**
		Early : Advanced	0.582	0.458-0.699	0.2399	90.48	59.18
	Sub -Type	**Control : ADC**	0.500	0.387-0.613	1.0000	50.00	40.00
		Control : SCC	0.524	0.402-0.644	0.8312	52.38	87.00
		ADC : SCC	0.570	0.427-0.705	0.3888	85.71	37.50
miR-375	**Stage**	**Control: NSCLC**	0.593	0.491-0.689	0.1618	57.41	85.00
		Control : Early	0.608	0.402-0.646	0.6211	53.85	74.00
		Control : Advanced	0.500	0.377-0.623	1.0000	52.63	0.00
		Early : Advanced	0.509	0.387-0.630	0.8958	100.00	41.18
	Sub-Type	**Control : ADC**	0.548	0.433-0.660	0.5943	54.84	67.50
		Control : SCC	0.690	0.569-0.796	0.0579	66.67	53.90
		ADC : SCC	0.574	0.431-0.709	0.3530	95.24	25.00

NSCLC patients were compared with normal samples as control and each other in seven ways. Significant values (<0.05) are shown in Bold. Number of samples is in control (n: 50), NSCLC (n: 72), early-stage NSCLC (n: 52), advanced-stage NSCLC (n: 20), ADC (n: 41) and SCC (n: 31)

been shown to exhibit oncogenic properties in ovarian and colorectal cancers, nasopharyngeal carcinoma, prostate cancer (PCa), classic papillary thyroid carcinoma, bladder cancer and NSCLC tissues, suggesting that plays dual roles in tumorigenicity and can modulate cellular motility and control stemness (37).

For the past five years, some studies demonstrated that the expression level of miR-141 in NSCLC tissues was higher than normal tissues (5,26,38–40). Liu et al. in 2012 reported that miR-141 expression was markedly up-regulated in NSCLC tissues, but interestingly there was no significant miR-141 dysregulation in paired serum samples (40). Nadal et al. in 2015 discovered that a hallmark up regulation of miR-141 in NSCLC tissues and sera in comparison with corresponding normal samples (38). Interestingly, Tejero et al. found that the correlation of miR-141 up-regulation and shorter overall

Table 4 AUC, sensitivity and specificity of miRNAs combinations.

Groups analysis (Control n: 50 / early NSCLC n: 52)					AUC	95% CI	P-value	Sensitivity	Specificity
Two miRNAs combination	**miR-21**	**miR-141**	**-**	**-**	**0.804**	**0.732-0.863**	**<0.0001**	**80.37**	**98.1**
	miR-21	mir-328	-	-	0.523	0.442-0.604	0.6268	55.14	85.3
	miR-21	miR-375	-	-	0.523	0.442-0.604	0.6268	51.4	73.0
	miR-141	**mir-328**	**-**	**-**	**0.727**	**0.650-0.795**	**<0.0001**	**72.22**	**92.2**
	miR-141	**miR-375**	**-**	**-**	**0.69**	**0.611-0.761**	**<0.0001**	**67.59**	**88.4**
	mir-328	miR-375	-	-	0.556	0.474-0.635	0.243	54.63	65.6
Three miRNAs combination	**miR-21**	**miR-141**	**mir-328**	**-**	**0.694**	**0.627-0.756**	**<0.0001**	**69.14**	**83.1**
	miR-21	**miR-141**	**miR-375**	**-**	**0.67**	**0.602-0.733**	**<0.0001**	**66.05**	**74.8**
	miR-21	miR-375	mir-328	-	0.506	0.437-0.576	0.8747	50	69.5
	miR-141	**mir-328**	**miR-375**	**-**	**0.62**	**0.551-0.686**	**<0.0001**	**61.11**	**79.9**
Four miRNAs combination	**miR-21**	**miR-141**	**mir-328**	**miR-375**	**0.623**	**0.561-0.681**	**0.0002**	**61.57**	**80.3**

Analysis of microRNAs combination among early NSCLC patients and controls clearly showed significant correlation in all groups that including miR-141 are shown in bold

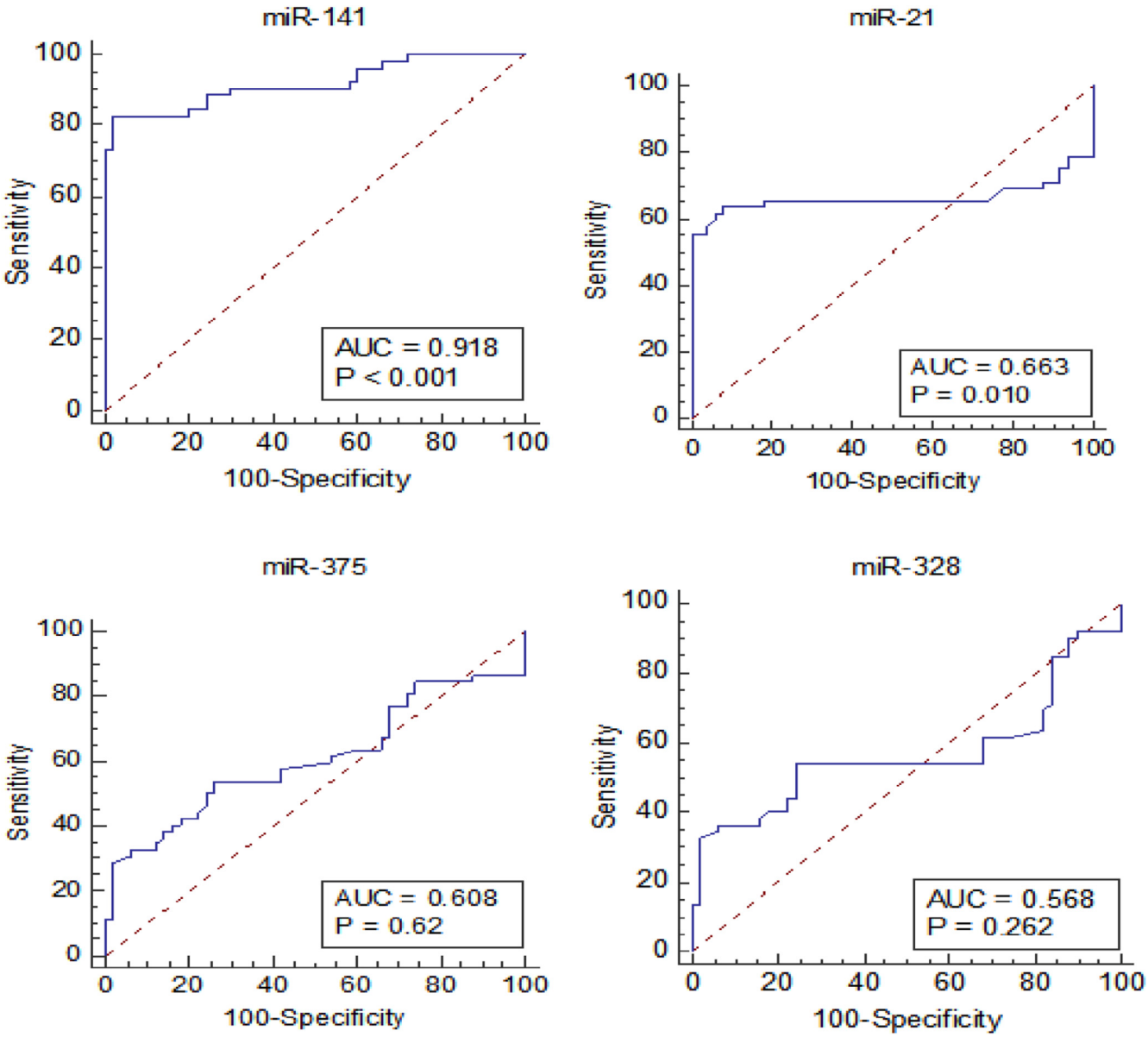

Figure 2 ROC curve analysis displaying diagnostic power of studied miRNAs in early stage of non-small lung cancer.

survival was only detected in adenocarcinoma, but not squamous cell carcinoma (39).

As considered, there is an inconsistency in circulating miR-141 expression level between Liu and our study may come from racial differences, sample differences (plasma vs serum), different of QPCR method (Taqman vs SYBR green), RNA isolation method (mirVana PARIS Kit vs miRNeasy), reference genes (U6 snRNA vs miR-24-3p) and determination of RNA concentration (NanoDrop technologies vs using spike-in RNA).

The potential impact of the angiogenesis mechanism was examined on the prognostic role of miR-141 in NSCLC. The *in vitro* angiogenesis role of miR-141 was related to reduction of KLF6 protein levels, increasing the secretion of VEGFA. It has been revealed that overexpression of miR-141 would lead to overproduction of VEGFA and increased neoangiogenesis in NSCLC (40). In addition, microRNA-141 directly targets PH domain leucine-rich-repeats protein phosphatase 1 (PHLPP1) and PHLPP2, which enhance the proliferation of NSCLC cells by promoting cell cycle progression (26). Gibbons et al. in 2009 reported that, miR-141 is highly more expressed in cell lines derived from primary tumors than cell lines derived from metastatic sites. It has been suggested that the metastatic process is associated with the down-regulation of miR-141 (41).

Over expression of miR-21 was reported repeatedly in several solid malignancies, including breast and lung cancer. In our previous studies, we reported that miR-21 is up-regulated in A549 cell line (42) and NSCLC tissues (43). It is mostly considered as an oncomiR which can alleviate apoptosis and increase proliferation through targeting tumor suppressor genes like BIM, PDCD4 and PTEN (44).

Our study reinforces the Wei et al. results (45), that plasma miR-21 was elevated in patients with NSCLC, particularly in

advanced stage, as compared to normal controls, and could be used to distinguish between tumors in advanced-stage and early-stage.

Ulivi et al. in 2013 showed that up-regulation of miR-328-3p of plasma could be used to correctly discriminate between patients with early NSCLC and healthy donors. While, in our investigation miR-328-3p had down regulation in advanced stage NSCLC patients versus normal (46).

Yu et al. in 2014 report that plasma miR-375 levels in NSCLC patients were significantly decreased in patient cohorts. In addition, patients with metastatic NSCLC had lower plasma miR-375 expression than those with non-metastatic NSCLC (47). But we did not find any significant correlation between miR-375 and NSCLC patients.

Furthermore, our results showed strong significance of miR-141 in distinguishing NSCLC patients from normal controls. Also, miRNA signature composed of 4 miRNAs (mir-141, miR-21, miR-328-3p and miR-375) showed strong discrimination power between patients and normal controls. However, the combination of these four miRNAs decreases the power of detection comparing miR-141 alone. Finally, we can suggest that plasma miR-141 is a worthy potential biomarker for early detection of NSCLC patients.

Conclusion

Our study strengthens that plasma miR-141 has the potential to be used as a cost effective, non-invasive screening test for non-small cell lung cancer. Further validation and optimizing improvement should be performed on larger sample to confirm our results.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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