Circulating miRNA-30a and miRNA-221 as Novel Biomarkers for the Early Detection of Non-Small-Cell Lung Cancer

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Abstract

Background: Detecting non-small-cell lung cancer at an early stage has become a great challenge due to the lack of a specific non-invasive marker. MicroRNAs are small, non-coding RNA molecules that play a role in carcinogenesis and cancer progression, as indicated by their abnormal expression in the patients' plasma. Herein, we investigated the plasma level of circulating miRNA-30a and miRNA-221 as non-invasive markers for an early detection of non-small-cell lung cancer.

Method: A cross-sectional study was conducted at Assiut University Hospital, Egypt, to investigate miRNA-30a and miRNA-221 expression via quantitative real-time PCR in the plasma of patients with non-small-cell lung cancer (n=70) and healthy controls (n=34). Receiver operating curves were used to evaluate the diagnostic value of miRNA-221 and miRNA-30a in non-small-cell lung cancer. The relationship between both markers and patient clinical parameters was further assessed.

Result: Circulating plasma miRNA-30a and miRNA-221 levels were significantly higher in the non-small-cell lung cancer patients compared with those in the healthy controls ($P<0.05$). There was a significant difference regarding the plasma miRNA-30a level among the three groups (the highest levels were recorded in adenocarcinoma, followed by large cell carcinoma and squamous cell carcinoma). ROC curve analysis of miRNA-30a and miRNA-221 showed that specificity and sensitivity were 60% and 80%, and 40% and 75%, respectively.

Conclusion: miRNA-30a and miRNA-221 may be non-invasive biomarkers for early detection and screening or therapeutic targets in patients with NSCLC. Future studies are warranted regarding the use of biomarkers as therapeutic targets.

Keywords: Lung cancer, mRNA-30a, miRNA-231, Non-small-cell lung cancer, Biomarker

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Introduction

Non-small-cell lung cancer (NSCLC), a common type of lung cancer, is among the most frequently diagnosed types of cancer and is the leading cause of mortality worldwide. Unfortunately, however, most patients with NSCLC are diagnosed at advanced stages.[1,2]

MicroRNAs (miRNAs) are small, noncoding RNA molecules found in eukaryotes that contain approximately 22 nucleotides and play negative roles in transcriptional and post-transcriptional gene expression regulation.[3] Some studies have concluded that more than 30% of proteins in the human body are regulated by miRNAs via several mechanisms.[4] Therefore, miRNAs may be important factors that not only regulate various biological processes, but also modulate carcinogenesis and the progression of diverse human cancers.[5] miRNAs are found to be stable in plasma and serum,[23] thereby representing an important role in clinical practice which may reduce unnecessary surgical interventions for early NSCLC diagnosis. These molecules are found to be higher in NSCLC tissues and cells compared with normal tissues and cells.[24]

MiRNA-221 is a member of the miRNA-221/222 family, reported to play a critical role in multiple cancers.[6] Down-regulation of miRNA-221 is associated with poor patient prognosis and drug resistance in NSCLC.[7, 8] MiRNA-30a, a member of the miRNA-30 family, is located on human chromosome 6q13 and has two mature forms: miR-30a-3p and miRNA-30a-5p.[8] Increasing evidence has confirmed the close relationship between the expression level of miRNA-30a and numerous types of human cancers; however, the exact role of miRNA-30a in cancer remains controversial. Some studies have suggested that miRNA-30a may act as a tumor suppressor, while others have suggested that it acts as an oncogene.[9,11,12,13]

This study was aimed to investigate the expression pattern of miRNA-30a and miRNA-221 in the plasma of NSCLC patients and to evaluate the potential of plasma miRNA-30a and miRNA-221 levels as novel tumor biomarkers for NSCLC diagnosis and prognosis.

Patients and Methods

In the present case-control study, blood samples were collected from 70 patients with NSCLC and 34 healthy control subjects after obtaining the approval of Institutional Research Board (IRB). All participants were recruited from the Chest and Clinical Oncology Departments, Assiut University Hospital, Assuit, Egypt, between
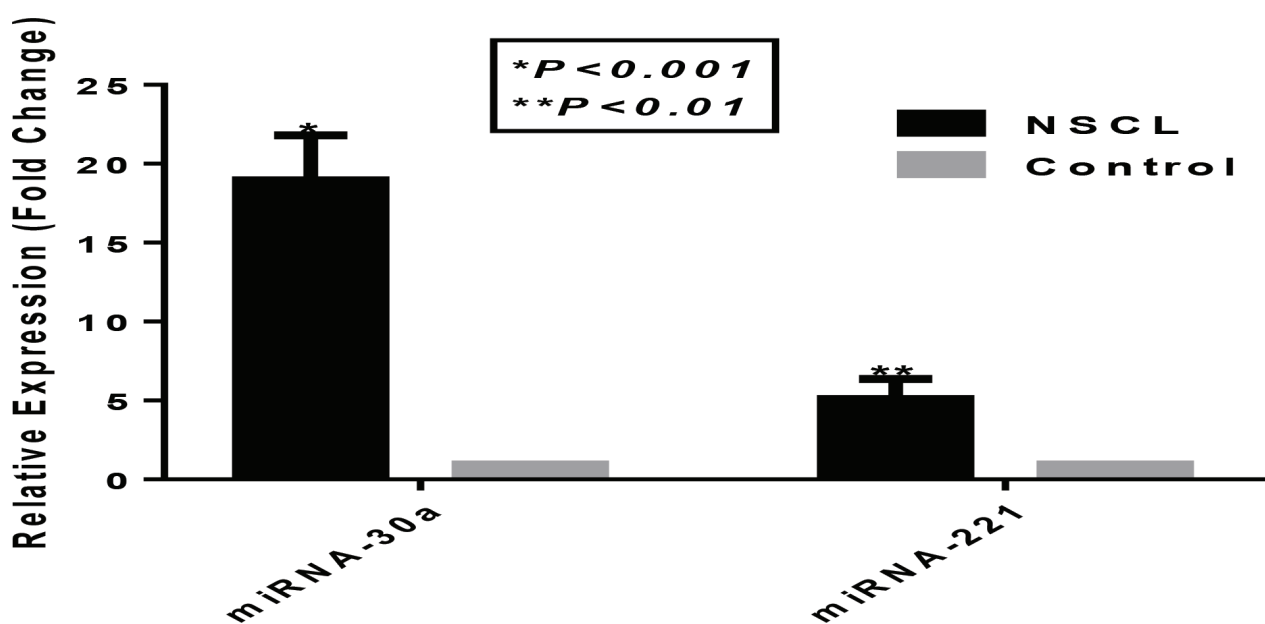

Figure 1. Fold change of miRNA marker among the studied patients.

Table 1. Socio-demographic and clinical data differences in NSCL cases vs. control

|	NSCL Cases (No.=70)	Control (No.=34)	*P*-value
Age in years	63.20 ± 5.7	55.60 ± 3.8	< 0.001*
Sex			
• Male	49 (70%)	17 (50%)	= 0.197**
• Female	21 (30%)	17 (50%)	
Smoking status			
• Non	11 (16%)	17 (50%)	= 0.018**
• Smoker	59 (84%)	17 (50%)	
Tumor pathological type			
• SCC	35 (50%)		
• Adenocarcinoma	24 (34%)		
• Large cell carcinoma 11 (16%)			
TNM staging			
• Stage II	28 (40%)		
• Stage III	42 (60%)		
LN metastasis			
• No	39 (56%)		
• Yes	31 (44%)		

*Independent t-test was used to compare the mean difference between groups; **Chi-square test was used to compare the percentages between groups

February 2015 and January 2017. Informed consent was further obtained from each patient and control prior to participation in the study. The lung cancer subjects included 35 patients with squamous cell carcinoma, 24 with adenocarcinoma and 11 with large cell carcinoma. These patients were at various clinical stages, including 28 at stage II of the disease and 42 at stage III according to the International Association for the Study of Lung Cancer Staging System. Additionally, the patients were classified according to TNM status: 31 with intrathoracic lymph node metastasis and 39 without metastasis (Table 1).

Exclusion criteria

Patients with pulmonary infections or TB, heart disease, hepatic or renal insufficiency, active bleeding, peptic ulcer, or malignancy elsewhere, and patients who received previous treatment for lung cancer were excluded from this study.
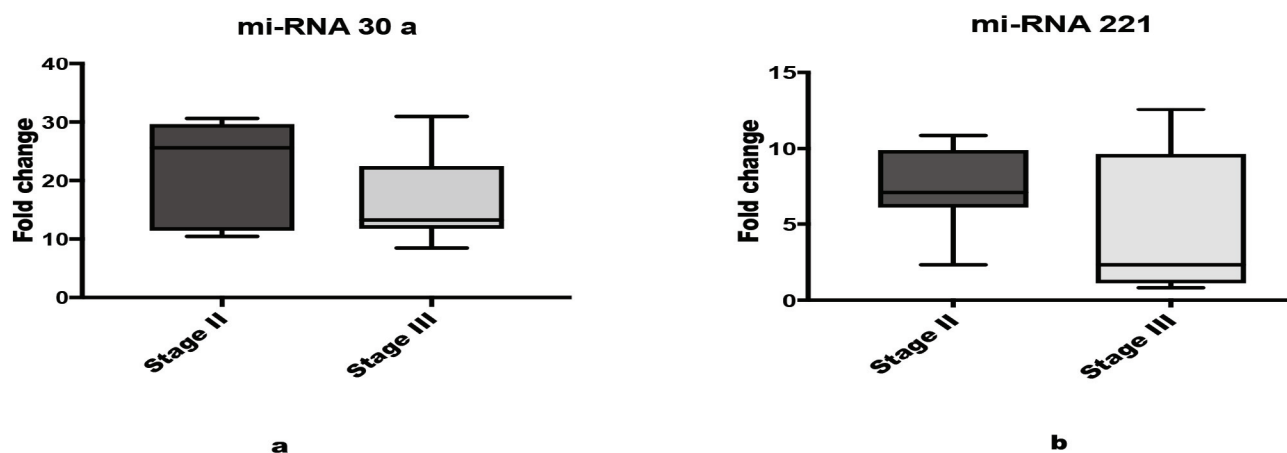
Figure 2. Mi-RNA 30a and mi-RNA 221 expression levels in NSCLC patients (n=70) with different TNM stages. (a) Plasma level of mi-RNA 30a in non-small lung cancer patients with different stages, (stage II, n=34) and (stage III, n=36). (b) Plasma level of mi-RNA 221 in non-small lung cancer patients with different stages, (stage II, n=34) and (stage III, n=36). *P*-values were calculated using unpaired t-test. Data are presented as "fold change".

Table 2. Biochemical parameters in NSCL cases and control

	NSCL Cases (No.=70)	Control (No.=34)	*P*-value*
• WBCs *109/L	6.51 ± 0.9	7.56 ± 0.5	= 0.297
• Hb (gm/dl)	10.05 ± 1.2	13.51 ± 1.7	< 0.001
• SGOT (U/L)	37.20 ± 3.8	31.80 ± 2.4	= 0.239
• SGPT (U/L)	31.55 ± 2.8	30.30 ± 1.8	= 0.754
• Serum Creatinine (mg/dl)	1.02 ± 0.1	0.95 ± 0.01	= 0.261
• Blood Urea (mg/dl)	36.25 ± 11.8	28.60 ± 7.0	= 0.017**
• PT Concentration %	78.59 ± 9.3	94.40 ± 8.3	< 0.001
• Blood PH	7.37 ± 0.1	7.40 ± 0.1	= 0.052
• Na (mmol/L)	132.77 ± 2.8	139.61 ± 2.2	< 0.001
• K (mmol/L)	3.77 ± 0.5	4.27 ± 0.4	= 0.007
• Platelet *109/L	166.50 ± 40.8	246.80 ± 15.2	= 0.004**
• miRNA-30A ct	23.67 ± 0.9	19.21 ± 1.1	< 0.001
• miRNA-221 ct	36.69 ± 1.9	35.56 ± 1.4	= 0.036

*Independent t-test was used to compare the mean difference between groups; **Mann Whitney U test was used to compare the median difference between groups

Plasma specimen collection

Five milliliters of venous blood were collected from each participant in the study into an EDTA-anticoagulant tube (BD, Franklin Lakes, NJ, USA) and centrifuged at 2000 rpm for 10 minutes. Plasma was transferred to a fresh tube and stored at 80°C until use.

RNA extraction and reverse transcription

Total RNA, including miRNA, was extracted from plasma using a Direct-zol™ RNA MiniPrep Kit (Zymoresearch, Catalog No. R2053, CA, USA). The RNA purity and concentration were determined using a Biotech Nanodrop system. Poly (A) polymerase enzyme (NEB, New England; Cat.no. M0276L) was used to increase the poly A tail of small noncoding miRNAs, and reverse transcription was conducted with a Thermo Scientific Revert Aid Reverse kit (Thermo, Waltham, MA, US). cDNA was collected following transcription.

qRT-PCR analysis of miRNAs

Quantitative real-time PCR (qRT-PCR) was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA) under the following conditions: hot-start step at 95°C for 7 min, followed by an initial denaturation for 20 sec at 95ºC, and annealing and extension for 60 sec at 59 ºC for 40 cycles. The relative miRNA-30a transcript levels were calculated via the following equation: fold change=$2^{-\Delta\Delta ct}$. U6-

3a

3b
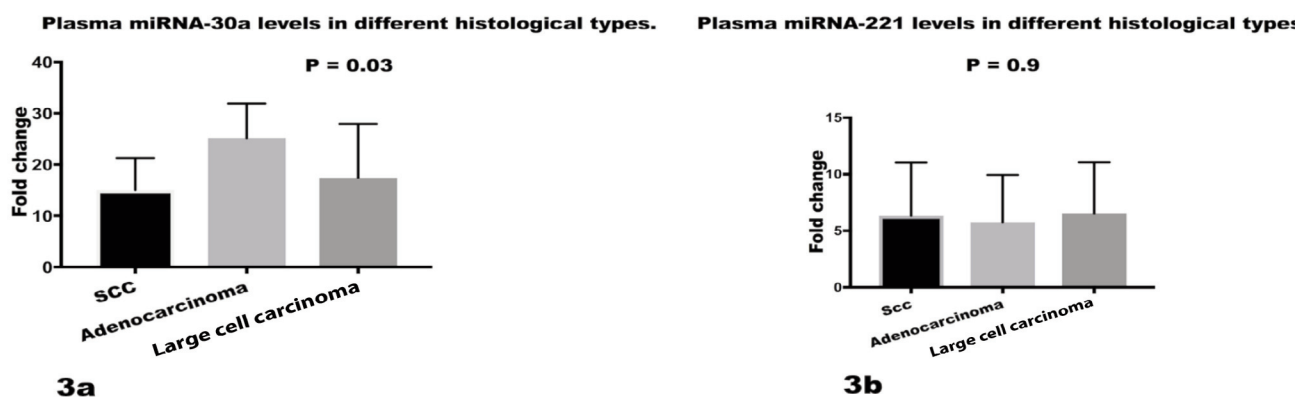
Figure 3. A significant difference was observed in the plasma level of mi-RNA 30a in non-small lung cancer patients with different histopathological types including (Scc, n=35) vs. (adeno carcinoma n=24) vs. (large cell carcinoma n=11) *P*=0.03*, while there was no significant difference in the plasma level of mi-RNA 221 in non-small lung cancer patients with different histopathological types including (squamous cell carcinoma, Scc, n=35) vs. (adeno carcinoma n=24) *vs.* (large cell carcinoma n=11) (*P*=0.9).

Table 3. Goodness criteria of miRNA biomarkers for the diagnosis of NSCLC

	Goodness criteria for miRNA Biomarkers	
	miRNA-30A	miRNA-221
AUC	0.625	0.668
Cut-off	27.75	34.84
Accuracy	70%	57.5%
Sensitivity, %	80%	75%
Specificity, %	60%	40%
PPV, %	66.7%	55.6%
NPV, %	75%	61.5%

*Sensitivity (true positives/all diseased); specificity (true negatives/all non-diseased); PPV (true positives/all test positives); NPV (true negatives/all test negatives).

snRNA acted as an internal control, and all primers were synthesized by RiboBio Co. (Guangzhou, China).

Statistical analysis

The collected data were verified, coded by the researcher, and analyzed using IBM-SPSS/PC/VER 21 and Graph Pad Prism 7 Software (San Diego, California, USA). Further calculated were the following descriptive statistics: mean, standard deviation, median, range, frequencies, and percentage. Test of significances: Chi square and Fisher Exact tests were used to compare the differences in the distribution of frequencies. Student t-test and Mann-Whitney U test were used to test the mean differences in continuous variables between groups (parametric and non-parametric). Using the 2-ΔΔCt method,

gene expression profile modulations were evaluated comparing Ct values between treated and non-treated cells. ROC curve depicted the diagnostic performance of NSCL biomarkers, analyzed as area under the curve (AUC), standard error (SE) and 95% CI. Validity statistics (sensitivity, specificity, and positive and negative predictive value, PPV and NPV) were further calculated. Significant test results were considered when P value was ≤ 0.05.

Sample size calculation

Sample size calculation was carried out using G*Power 3 software . A calculated sample of 100 respondents (70 NSCL cases and 30 controls) will be required to detect an effect size of 0.3 in NSCL biomarker levels, with an error probability of 0.05 and 90% power on a one-tailed test.
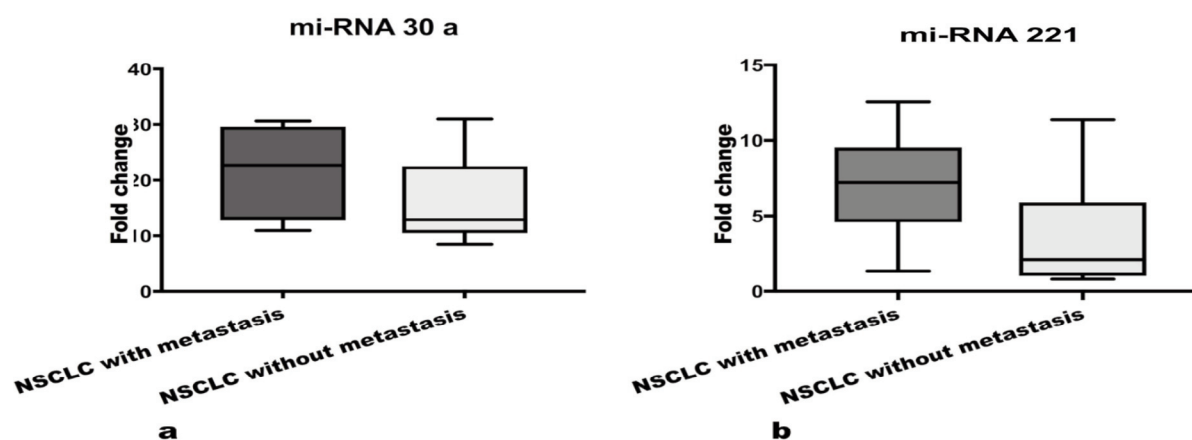
Figure 4. The plasma expression level of circulating mi-RNA 30a and mi-RNA 221 measured using qRT-PCR in (n=70) non-small cell lung cancer patients with different clinical status; (a) plasma level of mi-RNA 30a in non-small lung cancer patients with clinical status, (with metastasis, n=31) and (without metastasis, n=39). (b) plasma level of mi-RNA 221 in non-small lung cancer patients with different stages, (with metastasis, n=31) and (without metastasis, n=39). *P*-values were calculated using unpaired t-test. Data are presented as "fold change".

Results

Demographics

The minimum required sample was 100, and we recruited 104 patients. The demographic characteristics of the patient groups enrolled in the study are summarized in table 1.

The results showed a highly significant difference between the NSCLC patients (n=70) and the healthy controls (n=34) regarding the level of plasma miRNA-30a and miRNA-221 expression ($P<0.001$, $P=0.036$), (Table 2).

Plasma miRNA-30a and miRNA 221 levels in the NSCLC patients and controls

The results shown in figure 1 indicate that there was a significant difference in the level of plasma miRNA-30a and miRNA-221 expression between the NSCLC patients and controls.

Plasma miRNA-30a and miRNA 221 levels in the NSCLC patients with different clinical status

The results showed no significant difference concerning the level of plasma miRNA-30a and miRNA-221 expression between the NSCLC patients at stage II (n=28) and stage III (n=42) of the disease ($P=0.3$ and $P=0.1$, respectively), (Figure 2).

Plasma levels of miRNA-30a and miRNA-221 and histological types

As shown in figure 3a, there was a significant difference regarding the plasma miRNA-30a level among the three groups ($P=0.03$), the highest levels were recorded in adenocarcinoma, followed by large cell carcinoma and squamous cell carcinoma). However, the results showed no significant differences among these three groups ($P=0.9$) as regards the plasma miRNA-221 level (Figure 3b).

Plasma miRNA-30a and miRNA 221 levels in the NSCLC patients with different clinical status

Based on the results, there was an increase in the plasma level of miRNA-30a and miRNA-221 expression in the NSCLC patients at different clinical stages (with intrathoracic lymph node metastasis (n=31) versus without lymph node metastasis (n=39; $P=0.1$ and $P=0.057$). The difference was not statistically significant (Figure 4).

Diagnostic value analysis of miRNA-30a and miRNA-221 levels using receiver operating characteristic (ROC) curves

Table 3 and figure 5 show the cut-off values, sensitivity, specificity, predictive values and area under the curve for miRNA-221 and miRNA-
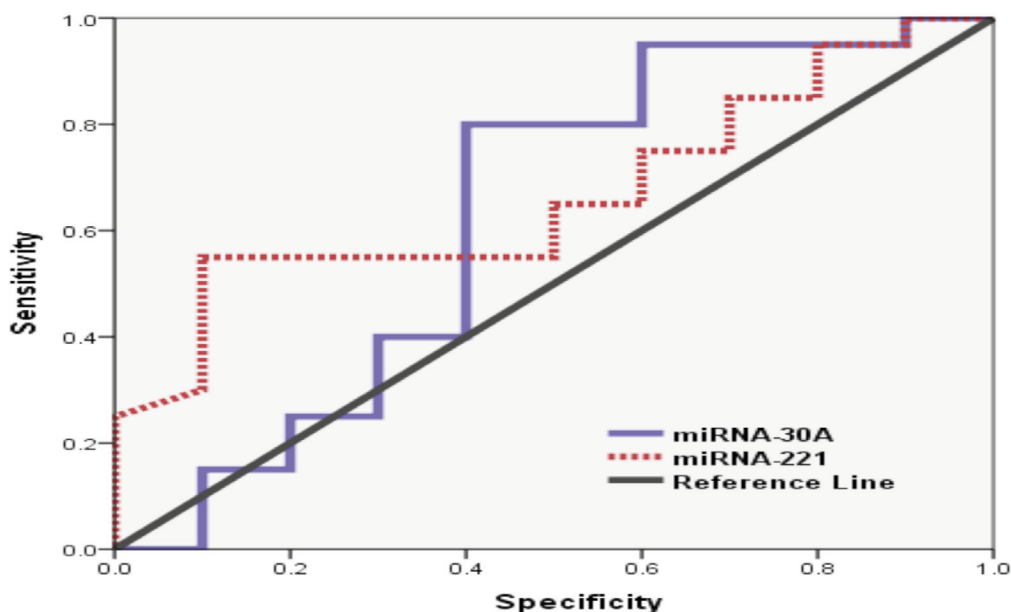

Figure 5. ROC curve of miRNA Biomarkers for the diagnosis of NSCLC.

30a. The best cut-off value of miRNA-221 was the index for NSCLC 34.84, with a sensitivity of 75% and a specificity of 40%. The best cut-off value of miRNA-30a was 27.75, with a sensitivity of 80% and a specificity of 60%, indicating that miRNA-30a has a more specific and sensitive biochemical diagnosis value (Figure 5).

Discussion

NSCLC is considered as the most common pulmonary carcinoma and a leading cause of mortality worldwide.[10] Identifying reliable blood biomarkers for early diagnosis and prognostic stratification of various human diseases is an area of intensive investigation.[11] Proteins, DNA, and mRNA can be detected in the circulatory system of cancer patients, and have been suggested in some studies to reflect the disease activity.[12] Owing to their stability and ease of detection, miRNAs have recently emerged as reliable biomarkers for disease status in several cancers and other diseases. miRNAs are endogenous, short non-coding RNAs that play a role in gene expression regulation by binding to their mRNA targets at both the coding and untranslated regions via base pairing with complementary sequences.[13,16]

The results of the present study demonstrated that the expression levels of circulating plasma miRNA-30a and miRNA-221 were significantly higher in the NSCLC patients compared with healthy controls. Regarding the miRNA-30a and miRNA-221 plasma levels, there was no significant difference between patients at stages II and III of non-small cell lung cancer. Results showed that the plasma miRNA-30a and miRNA-221 levels were higher (though not significantly) in the lung cancer patients with metastasis than in lung cancer patients without metastasis. There was a significant difference between squamous cell carcinoma, adenocarcinoma and large cell carcinoma patients concerning plasma miRNA-30a levels. However, in terms of miRNA-221 plasma levels, the difference between SCC, adenocarcinoma and large cell carcinoma patients was not statistically significant. ROC curve

analysis of miRNA-30a and miRNA-221 showed that the best cut-off value for miRNA-30a was 27.75, with a sensitivity of 80 % and a specificity of 60 %; on the other hand, the best cut-off value of miRNA-221 was 34.84, with a sensitivity of 60 % and a specificity of 40 %, meaning as a biomarker for detection of non-small-cell lung cancer, miRNA-30a was more specific and sensitive than miRNA-221.

Over the past few years, scientists have found enough evidence to confirm the aberrant miRNA expression between cancer patients and healthy individuals, strongly correlated with cancer development. In this regard, the results of our study provide additional support, as we observed a highly significant difference regarding miRNA-30a and miRNA-221 expressions between lung cancer patients and healthy controls. The present results agree with a previous study where the average miRNA-30a level dramatically increased in the plasma of NSCLC patients compared with those in both the benign control and the healthy groups. This suggests that miRNA-30a may be a useful biomarker for NSCLC diagnosis.[17] Additionally, a recent study reported a statistically significant difference in miRNA-221 expression between NSCLC patients and a healthy control group, further corroborating our results.[18] Collectively, this finding suggests that miRNA-30a and miRNA-221 may be employed as diagnostic biomarkers for NSCLC.

The present study indicated no significant difference between younger and older individuals regarding the plasma miRNA-30a and miRNA-221 levels, which is in accordance with a previous study where no significant difference was observed among NSCLC patients of different ages concerning miRNA-30a level.[17]

We further conducted stratified analyses of different clinicopathological subgroups according to smoking habits (smoker vs. non-smoker), tumor stage (stage II vs. stage III), and histological type (adenocarcinoma vs. squamous carcinoma vs. large cell carcinoma). No significant difference was seen in the plasma miRNA-30a and miRNA-221 levels of NSCLC patients at different TNM stages (stage II vs. stage III), suggesting their

important value in the screening and early detection of lung cancer, which might reduce the false-positive rate of computed tomography screening scans.[19] A recent study reported no significant difference between NSCLC patients at different clinical stages regarding the plasma miRNA-221 level, which provides additional support for the present results.[20]

Based on the histopathologic types of NSCLC, Sun et al. found no significant correlation between miRNA-30a level and NSCLC clinicopathological features, including histological type, age, gender, smoking history, TNM classification, lymph node metastasis, distant metastasis, and clinical stage.[17] Interestingly, the present study found that the plasma miRNA-30a level was significantly upregulated in the NSCLC patients with adenocarcinoma compared with other histological types (squamous cell carcinoma and large cell carcinoma), while no significant difference was found among the NSCLC patients with different histopathological types regarding plasma miRNA-221 level. More research is to be conducted on a larger number of patients in order to explain this result.

Our results further showed that the plasma miRNA-30a and miRNA-221 levels were (not significantly) higher in NSCLC patients with metastasis compared with NSCLC patients without metastasis. In agreement with this finding, Zheng and colleagues reported that the expression level of miRNA-221 was associated with lymph node metastasis. miRNA-221 has been suggested to promote NSCLC H460 cell growth[21] and inhibit the growth of NSCLC cell lines.[22] The specific underlying mechanism of miRNA-221 which affects NSCLC needs further studies.

A limitation of this study was the relatively small number of patients and the lack of follow-up to assess the plasma miRNA levels following different therapeutic modalities. More research is required to explain the exact oncogenic mechanisms of action in these markers.

In conclusion, this study revealed that miRNA-221 and miRNA-30a may be sensitive and specific biomarkers for early NSCLC diagnosis, and that increased circulating miRNA-30a and miRNA-221 expression levels may facilitate tumor progression. Therefore, miRNA-30a and miRNA-221 may be good prognostic biomarkers or therapeutic targets for patients with NSCLC.

Conflict of Interest

None declared.

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