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Original Article

Decreased plasma let-7c and miR-152 as noninvasive biomarker for non-small-cell lung cancer

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Abstract: Background: Non-small-cell lung cancer (NSCLC) is one of the leading causes of death. The aim of the present study was to compare the expression of let-7c and miR-152 in surgically resected NSCLC cases and healthy cases to evaluate their diagnostic impact. Methods: This hospital-based case-control study included 120 NSCLC patients and 360 healthy controls. The miRNA levels were measured via quantitative reverse transcription-polymerase chain reaction and their association with NSCLC was assessed by statistical data analysis and receiver operating characteristic curves. Results: The expression of let-7c and miR-152 in plasma were found to be downregulated in the patients with NSCLC. Advanced studies showed that the plasma let-7c and miR-152 were correlated with the clinicopathological features such as histological classifications, differentiation status, lymph node metastasis and stage classifications. The ROC curves for the miRNAs revealed a strong diagnostic performance. ROC curve analyses revealed that both plasma let-7c and miR-152 could serve as valuable biomarkers for NSCLC cases from healthy controls with an AUC of 0.714 and 0.845. Conclusion: It was found that let-7c and miR-152 are significantly reduced in plasma samples of NSCLC patients. These findings suggest that detection of circulating let-7c and miR-152 can be developed into a noninvasive and rapid diagnostic tool for the individuals with NSCLC.

Keywords: miRNA, non-small-cell lung cancer, diagnosis, biomarker

Introduction

Non-small-cell lung cancer (NSCLC) is one of the leading causes of death and annually responsible for more than 500,000 deaths worldwide [1]. The etiology of NSCLC still remains unknown. To date, although the main prognostic factor used in clinical practice is the tumor stage, several molecules and genetic alterations have also been reported as potential markers [2]. Nevertheless, prognosis of NSCLC depends on clinical-, genetic- and treatment- related factors [3]. Nowadays, a series of important biomarkers were found to be associated with the risk of NSCLC in studies with different designs and populations, which indicated that the genetic etiology of NSCLC was complicated, and the results needed further investigation in independent studies to confirm the associations with NSCLC risk [4].

MicroRNAs (miRNAs) are an abundant class of 17-25 nucleotides small non-coding RNAs and would post-transcriptionally regulate gene expression through directly binding to the 3' untranslational region (3' UTR) of target mRNAs [5, 6]. Till now, over 1000 kinds of miRNA have been identified in human spices, but revealing their roles in physiology and pathology is still an ongoing process. Recently, miRNAs have been suggested to participate in the regulation of diverse biological processes, and their deregulation or dysfunction plays important roles in cancer carcinogenesis and progression. However, deregulated miRNAs and their roles in cancer development remain largely illusive. Besides, more studies were conducted to evaluate the circulating miRNAs in early detection of NSCLC, and identified the differentially expressed miRNAs in NSCLC through different studies in recent years [7].

The let-7 family miRNA functions as a master regulator of cell proliferation pathways involved in cell cycle functions [8]. Decreased let-7c

Table 1. Primers used in qPCR

MiRNAs		Primer sequence (5'-3')
U6	Forward	GCTTCGGCAGCACATATACTAAAAT
	Reverse	CGCTTCACGAATTTGCGTGTCAT
let-7c	Forward	ACACTCCAGCTGGGTGAGGTAGTAGGTT
	Reverse	GGTGTCGTGGAGTCG
miR-152	Forward	ACTCTCGAGGCTTCTAAGCTGGGAACTTTGTC
	Reverse	ACTGAATTCCGCTTGTCTTGGACATATGGCACT

Table 2. Clinicopathological features of 120 NSCLC patients

Clinicialfetures	
Mean age (years)	63.2
< 60	65
≥ 60	55
Gender	
Male	72
Female	48
Tumor size	
0-3 cm	25
≥ .3 cm	95
Histological classification	
Adenocarcinoma	56
SCC	64
Differentiation	
Moderate-well	78
Poor	42
Lymph node metastasis	
Negative	68
Positive	52
Stage classification	
Stage I	69
Stage II, III, and IV	51

expression is linked to increased tumorigenesis and poor prognosis [9]. Let-7c was found to be down-regulated in several carcinoma cells in the large-scale microarray studies [10, 11]. A previous study based on the FFPE tissue showed that let-7c is down-regulated in metastatic tumor epithelial cells, and is up-regulated in the androgen-resistant tumors. It was further studied the expression pattern of let-7c in tumor-associated stroma cells and showed it is down-regulated in the stromal cells in tumors with extraprostatic extension. Together, these results support the role of let-7c as a general tumor suppressor gene [12, 13]. The expression of miR-152 is decreased in various tumor types, indicating that they have the potential to act as tumor-suppressor miRNAs. In a study of hepatic cell lines, it was found that miR-152 was downregulated in the liver cancer cell lines HepG2, MHCC97L, and MHCC97H relative to the hepatic cell line L02 [14]. Moreover, Huang et al. reported that miR-152 was downregulated in HBV-related HCC tissues compared with adjacent non-cancerous hepatic tissues [15]. In view of the above, it was speculated that miR-152 was important tumor suppressor gene for kinds of cancers. The aim of the present study was to compare the expression of let-7c and miR-152 in surgically resected NSCLC cases and healthy cases to evaluate their diagnostic impact.

Materials and methods

Ethics statement

This study has been approved by the Ethical Committee of Zhengzhou University. Written informed consents were obtained from all participants in this study as delineated by the protocol, which was also approved by the Ethical Committee of Zhengzhou University.

Study subjects

This hospital-based case-control study included 120 NSCLC patients and 360 healthy controls, and the informed consent was obtained. The 120 NSCLC cases were recruited from the First Affiliated Hospital of Zhengzhou University, Zhengzhou, China January 20012 and January 2014. They were all newly diagnosed, histopathologically confirmed and without a prior history of cancer or previous chemo- or radiotherapy. In total, 120 patients with NSCLC were recruited, all of whom were unrelated ethnic Han Chinese population (CHB). A detailed investigation of the clinicopathological data of NSCLC cases, including age, sex and et al, was conducted by trained interviewers through face-to-face interviews with the patients or the surgeons. The response to platinum-based (cisplatin or carboplatin) chemotherapy in patients with advanced NSCLC was assessed following the first two or three cycles and defined according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria 1.1 [16]. Follow-up was performed at least in the first three months from the time of operation.

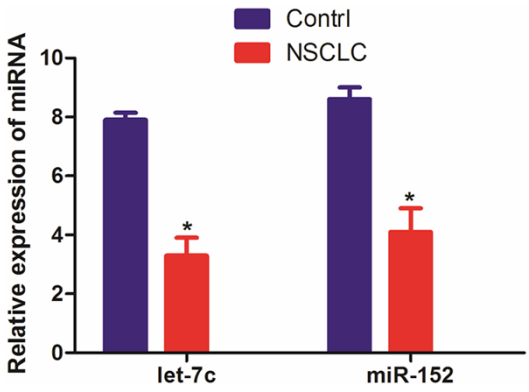

Figure 1. The relative of let-7c and miR-152 in NSCLC cases and controls.

RNA isolation

Total RNA was isolated from 0.9 to 2.1 mL plasma samples using a miRNAs isolation kit (TRI Reagent® BD, Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol minor modifiations. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA quality was measured using a denaturing 15% polyacrylamide gel. The reverse transcription reaction was carried out using the TaqMan.

Quantitative real-time RT-PCR

RNA isolated from plasma was diluted and used in 10 μL reverse transcription (RT) reactions using ABI (Applied Biosystems by Life Technology) miRNA-specific RT primers (Life Tech, Carlsbad, CA). For each miRNA and sample, RNA equivalent to 50 μL of biofluid was used in each RT reaction except for the probe sets for let-7c and miR-152, in which case RNA equivalent to 100 μL was used. The cDNA product was diluted 1:10 in water and 4.5 μL of the diluted product was combined in triplicate PCR-plate wells with 1 × ABI Universal PCR amplification mix and ABI miRNA-specific Taqman PCR primers in a final volume of 10 μL per well. The PCR plate was subjected to thermal cycling in an ABI StepOne Plus real-time PCR instrument. The cycling conditions were an initial incubation for 10 min at 95°C, followed by 40 cycles of 15 seconds at 95°C, 1 min at 60°C. The cycle threshold (CT) was detected using ABI software and a threshold of 0.05 was set. All the CT values were averaged to obtain the "mean CT value" in three independent detections. As shown in **Table 1**, the primers used in qPCR were presented.

Statistical analysis

For qRT-PCR data, the expression level of miRNA was normalized to U6 that was stable in serum samples. The mean for U6 was the same across all cohorts. The relative expression levels of each target miRNA (Log2 relative level) were calculated according to the difference in CT values between the target miRNAs and U6 by using the $-2^{\Delta\Delta CT}$ method. $-\Delta\Delta CT = (CT_{miRNA}-CT_{U6})_{cases}-(CT_{miRNA}-CT_{U6})_{controls}$. The U6 CT values were lower than the means of the other miRNA in this study. The ddH_2O were set up as negative controls of the in this study. Each sample was run in triplicate. Data are presented as the mean ± SD (standard deviation). Nonparametric Mann-Whitney test was used to compare difference in serum miRNA concentration between NSCLC cases and healthy controls. A *P*-value < 0.05 was considered statistically significant. For each miRNA, a receiver operating characteristic (ROC) curve was generated. The area under curve (AUC) value and 95% confidence intervals (CI) were calculated to determine the specificity and sensitivity of diagnosis of NSCLC. Statistical analysis was performed with IBM-SPSS software (Version 19).

Results

Clinicopathological features of NSCLC patients

The clinicopathological features of NSCLC patients were presented in **Table 2**. When recruited, the average age of all the NSCLC cases was 63.2 ± 9.6 years, respectively. Among all the cases, 65 cases were less than 60 years and 55 cases were over 60 years. A total of 72 males and 48 females were included in this study. When the tumor sizes were considered, 25 cases were in 0-3 cm group while 95 were in over 3 cm group. According to the histological classifications, 56 cases were adenocarcinoma and 42 cases were squarmouscellcarcinoma (SCC). According to the differentiation status, 78 cases were in moderate to well status while 42 cases were in poor differentiation status. Among all the cases, 68 cases were negative while 52 cases were positive. A total of 68 cases were positive in lymph node metastasis while 52 cases were negative. In the Stage I group, there were 69 cases while 51 cases in the Stage II to IV.

Table 3. Associations of aberrant miRNA expression with clinicopathological data of NSCLC patients

Clinicialfetures	N	let-7c		P	miR-152		P
		Low	High		Low	High	
Mean age (years)							
< 60	65	34	31	0.652	33	32	0.592
≥ 60	55	26	29		27	28	
Gender							
Male	72	36	36	0.573	34	38	0.542
Female	48	24	24		26	22	
Tumor size							
0-3 cm	25	12	13	0.532	14	11	0.267
≥ .3 cm	95	48	37		46	39	
Histological classifications							
Adenocarcinoma	56	38	18	0.001	25	31	0.180
SCC	64	22	42		35	29	
Differentiation status							
Moderate-well	78	29	49	< 0.001	30	48	0.001
Poor	42	31	11		30	12	
Lymph node metastasis							
Negative	68	28	40	0.021	33	36	0.648
Positive	52	32	20		27	25	
Stage classification							
Stage I	69	28	41	0.013	24	45	< 0.001
Stage II, III, and IV	51	32	19		36	15	

Associations of aberrant let-7c and miR-152 expression with clinicopathological data of NSCLC patients

In general, it was found that both let-7c (case vs control: 3.32 ± 0.76 vs 7.95 ± 0.78, P = 0.001) and miR-152 (case vs control: 4.12 ± 0.98 vs 8.60 ± 0.82, P = 0.014) were down-regulated in the plasma samples in NSCLC cases (**Figure 1**). We then investigated the associations between the altered miRNA expression levels (let-7c and miR-152 in plasma samples of NSCLC cases) with the clinicopathological characteristics of the NSCLC patients. We found that neither let-7c nor miR-152 expression were associated the age, gender and tumor size (P > 0.05). Advanced study showed that low expression of let-7c was strongly associated with NSCLC histology (P = 0.001, **Table 3**), whereas plasma miR-152 expression was not associated with histological classification. In contrast, both let-7c and miR-152 in plasma were associated the differentiation status (P < 0.001 and P = 0.001, respectively). Besides, we also found that both let-7c and miR-152 level were associated with the stage classifications (P = 0.013 and P < 0.001, respectively). However, it was also found that only the let-7c expression was associated with the lymph node metastasis (P = 0.021) rather than the miR-152 level (P = 0.648).

Diagnostic accuracy of plasma let-7c and miR-152 for NSCLC

A total of 120 NSCLC cases and 360 controls were involved in the detection of the diagnostic accuracy of plasma let-7c and miR-152. The ROC curve analysis was used to analyze the diagnostic accuracy of plasma let-7c and miR-152. ROC curve analyses revealed that both plasma let-7c and miR-152 could serve as valuable biomarkers for NSCLC cases from healthy controls with an AUC of 0.714 (95% CI: 0.523-0.718; P = 0.006) and 0.845 (95% CI: 0.732-0.962; P = 0.0002), respectively (**Figure 2A** and **2B**). At the cut-off value less than 3.12 for plasma let-7c, the sensitivity and the specificity were 72% and 78%, respectively. At the cut-off value less than 3.92 for plasma miR-152, the sensitivity and the specificity were 86% and %, respectively 81.3%.

The expression of let-7c and miR-152 in plasma between the pre-operative and post-operative in NSCLC

The post-operative plasma samples were obtained form the NSCLC cases in 96 cases of all the involved cases. It was found that the expression levels of let-7c (7.94 ± 1.32) in the post-operative plasmas from 96 cases were significantly increased when compared to the pre-operative paired plasmas (3.32 ± 1.26). Moreover, the post-operative plasma miR-152 expression was increased from 3.98 ± 1.08 to 7.98 ± 3.25 (**Figure 3**).

Discussion

Circulating miRNAs are quite stable even if in harsh conditions [17], and their expression levels are often altered in numerous respiratory diseases including NSCLC [18]. These make them ideal candidates for use as biomarkers for disease incidence and progression. Thus

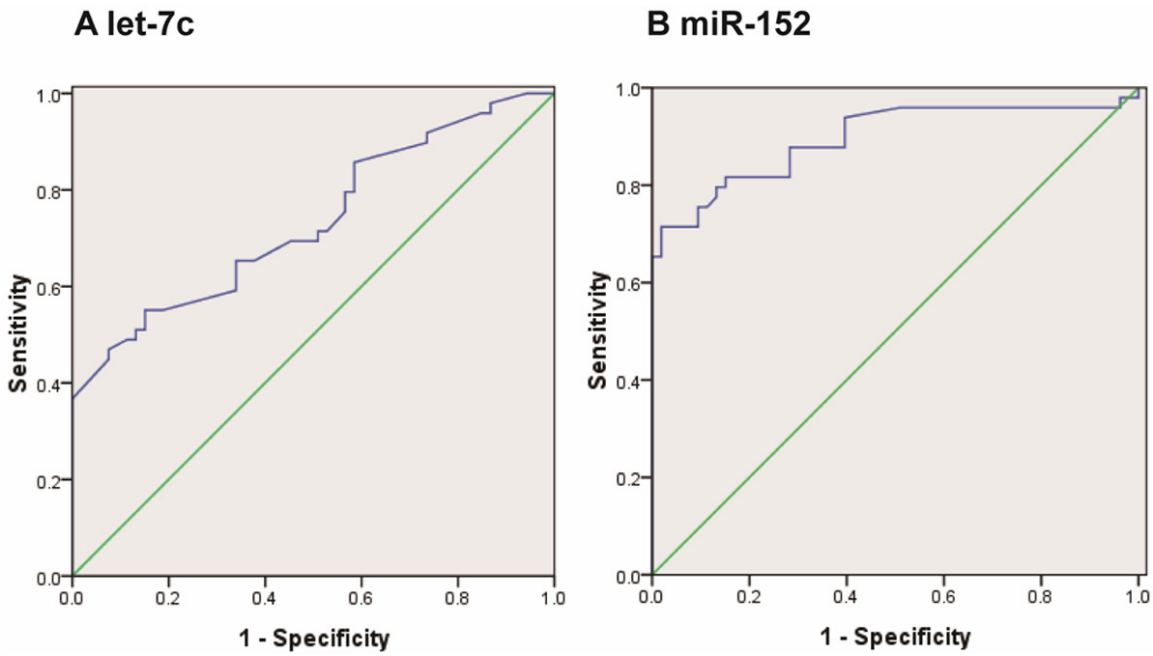

Figure 2. Diagnostic accuracy of plasma let-7c and miR-152 for NSCLC.

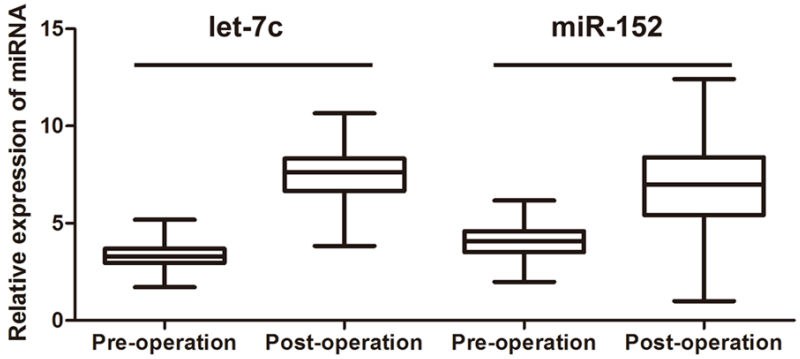

Figure 3. The expression of let-7c and miR-152 in plasma between the pre-operative and post-operative in NSCLC cases.

identification of the characteristically changed plasma miRNAs in the pathogenesis and progression of human diseases has been used to identify new blood-based biomarkers for disease diagnosis and prognosis. The NSCLC is a worldwide disease that causes cancer-related deaths of the patients. Currently, there are still no reliable biomarkers for the early diagnosis and the detection of disease progress of NSCLC.

In this observational study, we detected circulating let-7c and miR-152 expression by qRT-PCR. The expression of let-7c and miR-152 in plasma were found to be downregulated in the patients with NSCLC. Advanced studies showed that the plasma let-7c and miR-152 were corrected with the clinicopathological features such as histological classifications, differentiation status, lymph node metastasis and stage classifications. The ROC curves for the miRNAs revealed a strong diagnostic performance for each miRNA. ROC curve analyses revealed that both plasma let-7c and miR-152 could serve as valuable biomarkers for NSCLC cases from healthy controls with an AUC of 0.714 and 0.845. Besides, the post-operative expressions of both miRNAs were up-regulated and it indicated that the level of plasma let-7c and miR-152 might be used as prognosis biomarkers for NSCLC.

In this study, we found that let-7c was down-regulated in the NSCLC cases. Besides, low let-7c level was associated with poor differentiation status, lymph node metastasis and worse stage classification. Let-7c has been reported to be downregulated in pancreatic cancer, and in prostate cancer it has the ability to inhibit growth both in vitro and in vivo [19]. It was observed that let-7c had anti-proliferative prop-

erties through targeting the proliferation promoting transcription factor Myb which has been shown to be overexpressed in colon and breast cancer and other experimental data support the notion that let-7c has a negative effect on Myb mRNA and protein expression in different kinds of cancer cell lines. In previous in-vitro study, it was found that let-7c was downregulated in a NSLCL cell line, A549 cell with resistance to cisplatin compared with normal A549 cells. Advanced study showed that modulation of let-7c altered the sensitivity of A549 cells with resistance to cisplatin to cisplatin through regulating cisplatin-induced apoptosis. ABCC2 and Bcl-XL knockdown increased cisplatin sensitivity and cisplatin-induced apoptosis in A549 cells with resistance to cisplatin. While ABCC2 and Bcl-XL were identified as targets of let-7c, it suggested let-7c modulate cisplatin response in A549 with resistance to cisplatin cells through targeting ABCC2 and Bcl-XL [20]. In other study conducted in H1975 NSCLC cells. Fulvestrant increases the gefitinib sensitivity of H1975 cells and it was found that let-7c was most upregulated in the fulvestrant-treated cells. The results revealed that let-7c increases gefitinib sensitivity by repressing RAS and inactivating the phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways. In our study, we found that lower let-7c expression was associated with lymph node metastasis and it suggested that decreased let-7c might be associated with the migration or invasion. A study conducted by Zhao et al showed that overexpression of let-7c in relatively highly metastatic cells remarkably suppressed their migration and invasion. Inhibition of let-7c in cells with relatively low metastatic potential promoted their motility and invasion. Upon restoring the expression of ITGB3 and MAP4K3, the effects of let-7c on tumor metastasis were partially reversed, and more importantly, the expression levels of ITGB3 and MAP4K3 were inversely correlated with let-7c in 64 NSCLC tissues. In general, the repressor effect of let-7c in the NSCLC in quite credible.

The diagnostic value of plasma miR-152 for NSCLC cases was detected in a previous study. In a research with 52 patients with I-IIIA stages NSCLC, 10 patients with chronic obstructive pulmonary disease (COPD) and 20-age, sex and smoking status-matched healthy individuals, low miR-152 significantly predicted survival of squamous cell carcinoma patients [21]. The effect of miR-152 on the NSCLC has been discussed in several different studies. In the results from a in-vitro study, it showed that the expression of miR-152 was specifically downregulated in NiS-transformed cells via promoter DNA hypermethylation, whereas ectopic expression of miR-152 in NiS-transformed cells resulted in a marked reduction of DNMT1 expression. Further experiments revealed that miR-152 directly downregulated DNMT1 expression by targeting the 3' untranslated regions of its transcript. Interestingly, treatment of DNMT inhibitor would led to increased miR-152 expression by reversion of promoter hypermethylation, DNMT1 and MeCP2 binding to miR-152 promoter in NiS-transformed cells. Moreover, inhibition of miR-152 expression in 16HBE cells could increase DNMT1 expression and result in an increase in DNA methylation, DNMT1 and MeCP2 binding to miR-152 promoter, indicating an interaction between miR-152 and DNMT1 is regulated by a double-negative circuit. [22]. Su et al also found that we show that miR-152 is significantly downregulated in NSCLC tissues and cell lines. Restoration of miR-152 significantly reduces proliferation, colony formation, migration and invasion of NSCLC cells. In addition, ADAM metallopeptidase domain 17 (ADAM17) is identified as a target of miR-152 in NSCLC cells, and miR-152-induced suppression of cell proliferation, colony formation, migration and invasion is partially mediated by silencing of ADAM17 expression. Furthermore, ADAM17 inversely correlates with miR-152 in NSCLC tissues [23]. Another study was conducted to investigate whether the CpG island methylation of certain microRNAs was associated with the clinicopathological features and the prognosis of non-small-cell lung cancer. The results showed that the methylation of mir-152 was analyzed in 96NSCLC specimens using a combined bisulfite restriction analysis. The median observation period was 49.5 months. The methylation ofmiR-152 was individually associated with an advanced T factor independent of age, sex, and smoking habit. Moreover, the methylation of this miRNA locus was associated with a poorer progression-free survival in a univariate analysis [24].

In our study, we found that the let-7c and miR-152 were up-regulated in the post-operative

samples. This findings showed the association of the levels of there miRNAs were associated with the cancers directly. Although our results are promising, there still are some limitations in this study. Without a follow-up study, it is hard for us to detect the prognostic value for there miRNAs. Advanced follow-up study with more acceptable samples would be conducted for this issue.

In conclusion, we have shown that let-7c and miR-152 are significantly reduced in plasma samples of NSCLC patients. These findings suggest that detection of circulating let-7c and miR-152 can be developed into a noninvasive and rapid diagnostic tool for the individuals with NSCLC. Furthermore, screening a large cohort of plasma samples of susceptible subjects will further determine the usefulness of let-7c and miR-152 expression as a potential pre-NSCLC biomarker.

Disclosure of conflict of interest

None.

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