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RESEARCH ARTICLE

Serum miR-152, miR-148a, miR-148b, and miR-21 as novel biomarkers in non-small cell lung cancer screening

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Abstract Lung cancer, predominantly by non-small cell lung cancer (NSCLC), is the leading cause of cancer-related deaths over the world. Late diagnosis is one of important reasons for high mortality rate in lung cancer. Current diagnostic approaches have disadvantages such as low accuracy, high cost, invasive procedure, etc. MicroRNAs were previously proposed as promising novel biomarkers in cancer screening. In this study, we evaluated the predictive power of four candidate miRNAs in NSCLC detection. Our study involved 152 NSCLC patients and 300 healthy controls. Blood samples were obtained from the total 452 subjects. After miRNA extraction from serum, the expression of miRNAs in cases and controls were quantified by qRT-PCR and normalized to the level of U6 small RNA. Statistical analyses were performed to compare miRNA levels between cases and controls. Stratified analyses were employed to compare miRNA levels in NSCLC patients with different clinical characteristics. Serum miR-148a, miR-148b, and miR-152 were significantly downregulated in NSCLC patients. However, overexpression of serum miR-21 was observed in NSCLC patients. The combination of four candidate miRNAs exhibited the highest predictive accuracy in NSCLC screening compared with individual miRNAs (AUC=0.97). Low level of miRNA-148/152 members may associate with advanced stage, large tumor size, malignant cell differentiation, and metastasis. High expression of miR-21 was possibly correlated with large size tumor and advanced cancer stage. Our results showed the dysregulation of miR-148/152 family and miR-21 in NSCLC patients. Hence, the four candidate miRNAs have great potential to serve as promising novel biomarkers in NSCLC screening. Further large-scale studies are needed to validate our results.

Keywords MicroRNAs · miR-148a · miR-148b · miR-152 · miR-21 · Serum · Non-small cell lung cancer · Screening

Introduction

Lung cancer is one of the most prevalent cancers and the leading cause of cancer-related deaths in China and worldwide. It was estimated that approximately 1.82 million new cases of lung cancer were diagnosed around the world in 2012 [1]. Non-small cell lung cancer (NSCLC) is a predominant type of pulmonary carcinoma, which accounts for 85 % of all lung cancer cases [2]. Despite the advances over the past decades in terms of cancer treatments, the overall five-year survival rate for NSCLC patients remains less than 15 % [3]. Late diagnosis of NSCLC is one of the important contributing factors for dismal clinical outcome since the tumor have probably spread to distant organs at the time of diagnosis. Besides, there was no practical approach available at present for NSCLC screening in the population at risk. Therefore, the challenge lies in how to accurately and cost-effectively identify the individuals at early stage NSCLC. Early diagnosis of

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NSCLC facilitates the timely intervention to suppress tumor growth.

Conventional diagnostic methods, such as computed tomography (CT), positron emission tomography (PET), and X-ray, are widely applied in the NSCLC diagnosis. However, these approaches have several limitations. False positive rate is relatively high in CT screening, and benign lung nodules can be possibly misdiagnosed as malignant tumors [4]. PET scan requires an intravenous injection of radionuclide for the procedure. It might cause some discomfort or possible allergic reaction. X-ray may pose a potential risk of radiation to patients, especially for those who need regular examination. Thus, it is urgently needed to develop accurate and non-invasive methods to detect early stage NSCLC.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate a wide range of genes in post-transcriptional level. MiRNAs bind to the 3′-untranslated region (3′-UTR) of messenger RNA (mRNA), leading to translational repression or mRNA degradation. The genes regulated by miRNAs were implicated in various physiological processes. Thus, miRNAs play important role in diverse biological activities as well, such as cell proliferation, differentiation, and apoptosis [5]. Emerging studies have demonstrated that miRNAs were aberrantly expressed in cancer patients, which were proposed as predictive indicators for human cancers. Besides, miRNAs was ubiquitously expressed in blood, urine, and feces with high stability. Collectively, miRNAs were shown to have great clinical value in cancer screening.

Concerning NSCLC, Yu et al. identified seven miRNAs aberrantly expressed between NSCLC cases and healthy controls. Among the seven miRNAs, miR-486, miR-126, and miR-145 were downregulated while miR-21, miR-182, miR-375, and miR-200b were upregulated in NSCLC patients [6]. MiR-126 represses the tumor growth by targeting vascular endothelial growth factor A and v-crk sarcoma virus CT10 oncogene homologue (CRK) [7]. Zhu et al. found differential expression of three miRNAs (miR-29c, miR-93, and miR-429) in NSCLC tissues compared to corresponding non-malignant lung tissues and suggested that the three miRNAs have clinical value in the diagnosis and prognosis of NSCLC [8]. Decreased level of miR-29 results in gene silencing and aberrant methylation by activating the methyltransferase genes DNA (cytosine-5-)-methyltransferase 3 alpha and beta (DNMT3A, DNMT3B), ultimately leading to tumorigenesis [9]. Tang et al. showed that aberrant plasma level of miR-21, miR-145, and miR-155 were associated with lung cancer and the panel of miR-21, miR-145, and miR-155 exhibited a higher diagnostic accuracy in lung cancer detection [10]. MiR-145 was revealed to inhibit Akt activation to suppress the lung cancer cell growth [11]. Nonetheless, little was known about the association between expression of miR-148/152 family members and NSCLC. MiR-21 is one of the most studied miRNAs, which has been studied in various cancers. Therefore, we selected miR-148/152 family along with putative oncogene miR-21 to investigate their predictive performance in NSCLC in this study.

MiR-148/152 family members include miR-148a, miR-148b, and miR-152, of which aberrant expression was observed in a series of diseases, such as diabetes [12], atherosclerosis [13], IgA nephropathy [14], and cancers [15]. In previous studies, miR-148b was downregulated in liver stem cells [16]. Low level of miR-148a was associated with large tumor size and advanced gastrointestinal cancer stage [17]. Song et al. reported a significantly low expression of miR-148b in gastric cancer tissues and cell lines. Additionally, CCKBR, regulatory peptides of brain and gastrointestinal tract, is the target of miR-148a and miR-148b. Dysregulation of CCKBR may result in the gastrointestinal and gastric tumors [18].

The objective of our study is to assess the predictive performance of serum miR-148a, miR-148b, miR-152, miR-21, and their group in NSCLC screening. We compare the serum level of miRNAs between NSCLC patients and healthy controls.

Materials and methods

NSCLC patients and healthy individuals

The protocol of this study was approved by Ethics Committee of Central Hospital of Zaozhuang Mineral Group, and the study was performed in strict compliance to the Declaration of Helsinki. Written informed consents must be provided by each participant prior to the beginning of our study.

All 452 participants, including 300 NSCLC patients and 152 healthy controls, were recruited between July 2011 and October 2013 from Central Hospital of Zaozhuang Mineral Group. The recruitment of NSCLC patients had to meet the criteria as follows: 1) the diagnosis of NSCLC must be confirmed by histopathological examination; 2) patients with previous cancer history were excluded in our study; and 3) NSCLC patients were not given the chemotherapy or radiotherapy before. The TNM staging system was used to determine the stage of NSCLC in patients at the time of diagnosis. The differentiation and histological types of malignant cells were assessed by morphological and histological examination. A summary of the clinical and pathological information for all cases and controls were presented in Table 1.

Five milliliter peripheral blood was obtained from each participant. Blood specimens were centrifuged at 3000 rpm for 10 min, followed by another centrifugation at 12,000 rpm for 5 min, to separate the serum from whole blood. Serum specimens were stored −80 °C before processing.

Table 1 Clinicopathological characteristics of NSCLC patients and healthy controls

Clinical characteristic	NSCLC (n=152)	Control (n=300)	P value
Age			
≤60 (%)	46 (30.3 %)	112 (37.3 %)	0.294
>60 (%)	106 (69.7 %)	188 (62.7 %)	
Gender			
Male (%)	98 (64.5 %)	206 (68.7 %)	0.339
Female (%)	54 (35.5 %)	94 (31.3 %)	
Smoking status			
Current (%)	51 (33.6 %)	86 (28.7 %)	0.149
Former (%)	77 (50.6 %)	153 (51.0 %)	
Never (%)	24 (15.8 %)	61 (21.3 %)	
TNM stage			
Stage I–II (%)	34 (22.3 %)	–	–
Stage III–IV (%)	118 (77.6 %)	–	
Differentiation			
Poor (%)	55 (36.2 %)	–	–
Moderate-well (%)	97 (63.8 %)	–	
Lymph node metastasis			
Negative (%)	25 (16.4 %)	–	–
Positive (%)	127 (83.6 %)	–	
Histological type			
Adenocarcinoma (%)	57 (37.5 %)	–	–
Squamous carcinoma (%)	74 (48.7 %)	–	
Others (%)	21 (13.8 %)	–	

RNA extraction

Total RNA were extracted from 400 μl serum by using mirVana PARIS Kit (Ambion, Austin, TX, USA). The extraction was carried out according to the manufacturer's protocol. Extracted RNAs were eluted by 50 μl RNase-free water (Ambion, Austin, TX, USA). The quality of extracted RNAs was examined by measuring the absorbance at 260/280 nm and 260/230 nm on NanoDrop 1000A spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Quantification of miRNAs by qRT-PCR

Quantification of miRNAs was performed using human TaqMan MicroRNA Assay Kits (Applied Biosystems, Foster City, CA, USA). Reaction of complementary DNA (cDNA) synthesis was conducted in 15 μl volume containing 5 μl of RNA extract, 3 μl of reverse transcription primers, 0.15 μl of 100 mM dNTPs, 0.19 μl of 20 U/μl RNase Inhibitor, 1.5 μl of 10× reverse transcription buffer, 1 μl of 50 U/μl Multiscribe Reverse Transcriptase, and 4.16 nuclease-free water. Reaction mixture was incubated at 16 °C for 30 min, 42 °C for 30 min, 85 °C for 30 min, and terminated at 4 °C. After the synthesis of cDNA, PCR mixtures in 20 μl volume including 1.33 μl cDNA, 10 μl of TaqMan Universal PCR Master Mix II (2×) with no UNG reagent (Applied Biosystems, Foster City, CA, USA), 1 μl of specific primers, and 7.67 μl of RNase-free water were prepared. The qRT-PCR conditions were 10 min initial denaturation at 95 °C followed by 45 cycles of amplification at 95 °C for 15 s and at 60 °C for 60 s. Cycle threshold (Ct) values were analyzed by Bio-Rad IQ5 2.1 Standard Edition Optical System Software 2.1.94.0617. Expression of miRNAs was normalized to expression of U6 small RNA (RNU6), which served as internal controls. RNU6 is widely used as endogenous reference since RNU6 have a stable and constant expression under experiment conditions and also similar abundance to candidate miRNAs. Fold difference in miRNA expression was expressed as $-\Delta Ct$, which was the difference of Ct values between miRNAs and U6 small RNA.

Statistical analysis

Differences in miRNA levels between cases and controls were assessed by student's t test. Chi-square test and one-way ANOVA were used to assess the difference in clinicopathological characteristics and association between miRNA levels and clinicopathological characteristics between cases and controls. A two-sided P value less than 0.05 was regarded as statistically significant. Receiver operating characteristics (ROC) curves and the area under the curve (AUC) were plotted to analyze the sensitivity, specificity, and cutoff value of miRNAs. The predictive performance of miRNA panel was evaluated using logistic regression analyses. Statistical

analyses were carried out with STATA version 12.0 software (Stata Corp, College Station, TX, USA), and the graphs were generated by using GraphPad Prism 5.0 (Graphpad Software Inc., San Diego, CA, USA)

Results

Clinical characteristics of study population

As shown in Table 1, no significant variation in age and gender was observed between NSCLC patients and healthy controls. Smoking rate, including regular and former smokers, was significantly higher in NSCLC group compared with healthy controls. In NSCLC patients, more than three quarters of cases were diagnosed as advanced-stage NSCLC. Under the morphological observation, approximately 64 % of patients have moderately and well-differentiated malignant cancer cells. The lymph node metastasis was found in nearly 84 % of cases. In view of histological type, the total of 152 NSCLC cases can be categorized into adenocarcinoma (n=57), squamous carcinoma (n=74), and others (n=21).

Serum levels of miRNAs in NSCLC patients and healthy controls

To investigate the expression patterns of four candidate miRNAs (miR-148a, miR-148b, miR-152, and miR-21) in NSCLC patients and healthy controls, expression of four miRNAs were examined in serum samples collected from 152 NSCLC patients and 300 healthy controls by qRT-PCR. As shown in Fig. 1a, serum levels of miR-148a, miR-148b, and miR-152 were significantly lower in NSCLC patients (P<0.001) compared with healthy controls. In contrast, overexpression of miR-21 was found in NSCLC patients (P<0.001), which was consistent with previous studies.

Predictive power of miRNAs in NSCLC detection

ROC curves were illustrated in Fig. 1b and their AUC values were calculated to further evaluate the predictive performance of candidate miRNAs. All selected individual miRNAs showed a relatively high predictive accuracy, and their AUC values were 0.90 (95 % confidence interval (95 %CI), 0.86–0.95; sensitivity=85 %; specificity=83 %) for miR-148a, 0.90 (95 % CI, 0.87–0.95; sensitivity=83 %; specificity=83 %) for miR-148b, 0.82 (95%CI, 0.76–0.87; sensitivity=75 %; specificity=77 %) for miR-152, and 0.81 (95 % CI, 0.73–0.85; sensitivity=69 %; specificity=71 %) for miR-21. The panel of miR-148a, miR-148b, miR-152, and miR-21 had the highest predictive accuracy in NSCLC screening with its AUC value of 0.98 (95 % CI, 0.95–0.99; sensitivity=96 %; specificity=91 %).

Association between miRNAs expression and clinical characteristics in NSCLC patients

Stratified analyses were conducted to further assess the miRNA level in NSCLC patients with different clinical characteristics. The results of stratified analyses were presented in Table 2. First, we calculated the average level of each miRNA in 152 NSCLC patients. The NSCLC patients were categorized based on the different clinical features. Moreover, the levels of miRNAs in each patient were compared with the corresponding average miRNA levels of total 152 patients. If the miRNA level of a patient was higher than the average level, it was determined as “high”. Otherwise, it was regarded as “low”. The number of patients was counted according the clinical characteristics and miRNAs levels.

Our results suggested that patients with tumor larger than 3 cm appeared to have lower expression of miR-148a and in contrast higher expression of miR-21. Similarly, downregulation of miR-148b and upregulation of miR-21 were observed in patients at stage III or IV of NSCLC. Additionally, decreased level of miR-152 tended to associate with the

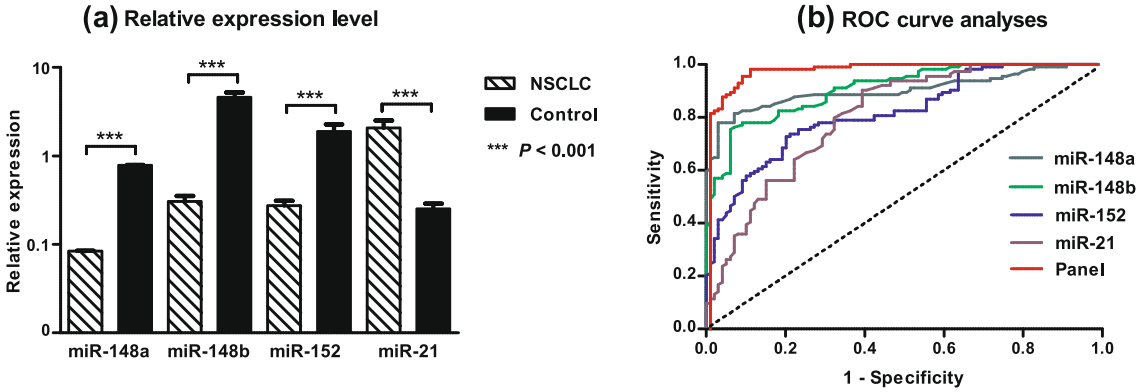

Fig. 1 Predictive poewer of miRNAs in differentiating NSCLC patients from healthy controls. **a** Relative expression levels of miRNAs in NSCLC patients and healthy controls. **b** ROC curve analysis of miRNAs in differentiating NSCLC patients from healthy controls

Table 2 NSCLC patients' characteristics and correlation of relative levels of miRNAs with clinic pathological parameters

Characteristics	No.	miR-148a			miR-148b			miR-152			miR-21		
		Low	High	*P* value	Low	High	*P* value	Low	High	*P* value	Low	High	*P* value
Age													
≤60	46	24	22	0.571	23	23	0.915	22	24	0.806	20	26	0.167
>60	106	50	56		52	54		53	53		59	47	
Gender													
Male	98	53	45	0.630	52	46	0.562	53	45	0.792	44	54	0.298
Female	54	27	27		26	28		28	26		29	25	
Tumor size													
0–3 cm	39	15	24	*0.014*	17	22	0.405	19	20	0.853	29	10	*0.001*
>3 cm	113	69	44		58	55		57	56		50	63	
TNM stage													
Stage I–II	34	20	14	0.179	10	24	*0.006*	18	16	0.515	24	10	*0.004*
Stage III–IV	118	54	64		66	52		55	63		50	68	
Differentiation													
Poor	55	24	31	0.121	25	30	0.276	23	32	*0.024*	27	28	0.249
Moderate-Well	97	55	42		53	44		59	38		57	40	
Lymph node metastasis													
Negative	25	8	17	*0.020*	11	14	0.308	12	13	0.562	13	12	0.998
Positive	127	73	54		70	57		69	58		66	61	
Histological type													
Adenocarcinoma	57	28	29	0.993	29	28	0.800	29	28	0.981	27	30	0.810
Squamous carcinoma	74	36	38		34	40		37	37		39	35	
Others	21	10	11		11	10		11	10		10	11	

moderate or well differentiation of malignant cells. Furthermore, NSCLC patients with lymph node metastasis have a lower expression of miR-148a. MiRNA levels have no apparent correlation with age, gender, and histological types.

Discussion

In the present study, we found that miR-148a, miR-148b, miR-152, and miR-21 were significantly dysregulated in NSCLC patients compared with healthy controls. MiR-148a, miR-148b, and miR-152 were markedly downregulated in patients. However, miR-21 was notably upregulated in patients, which was consistent with previous findings. Our results indicated that miR-148a, miR-148b, and miR-152 served as tumor suppressors, and miR-21 acted as an oncogene in the carcinogenesis. The panel of four candidate miRNAs showed the highest predictive accuracy in NSCLC detection (AUC= 0.98). Stratified analyses indicated that low level of miR-148a was associated with large size tumor and lymph node metastasis. Furthermore, low level of miR-148b and miR-152 was correlated with advanced NSCLC stage and moderate well differentiation of malignant cells. On the contrary, high level of miR-21 was associated with large size tumor and advanced cancer stage. Collectively, miR-148a, miR-148b, miR-152, and miR-21 presented great clinical value in NSCLC preliminary screening, and further studies in a large population may be needed to validate the feasibility of these miRNAs as novel non-invasive biomarkers.

Over the past decades, miRNAs as non-coding RNAs were proven to regulate gene expression by repressing the translation or cleavage of various mRNAs. Besides, miRNAs were reported to function as oncogenes or tumor suppressors and play important role in the development and progression of cancers, which could provide new therapeutic targets for cancer treatment or screening without any invasive procedures involved. Actually, a number of studies have already proposed that circulating miRNAs can be used as novel biomarkers for cancer screening, such as prostate cancer [19], breast cancer [20], colorectal cancer [21], lung cancer [22], and gastric cancer [23]. However, the molecular mechanism of miRNAs implicated in carcinogenesis still remains unclear since miRNAs regulates a wide range of downstream genes, which participate in complex regulatory pathways.

In regard to NSCLC, there have been increasing researches reporting the dysregulation of miRNAs in NSCLC patients.

It's been demonstrated that a number of miRNAs may participate in the tumorigenesis, surivival, angiogenesis, invasion, and metastasis of lung cancer (Fig. 2). Low expression of let-7 is one of extensively studied and well-established contributing factor to lung cancer. Moreover, high expression of let-7 inhibits malignant cell growth in lung adenocarcinoma cell line [24, 25]. Besides, the single nucleotide polymorphism in the 3′UTR of K-Ras gene, the binding site of let-7, was correlated with higher risk of NSCLC [26]. The tumor-suppressive role of let-7 was also supported by the fact that it inhibits the important cell cycle regulatory such as cdk6 and cdc25 [27]. Other miRNAs have also been investigated their roles in lung cancer. MiR-21 is another most studied molecule, which was also investigated in our study. It is also shown to function as oncogenes in various cancers. Capodanno et al. previously revealed that miR-21 expression was significantly increased in NSCLC tissues, which can be used as to distinguish the NSCLC from noncancerous lung tissues [28]. Furthermore, expression of miR-21 is upregulated by activation of EGFR signaling pathway in lung cancer development [29]. Additionally, miR-21 represses the expression of tumor suppressor PTEN in post-transcriptional level and invokes NSCLC the development and invasion [30].

MiR-148/152 family was found to be decreased in a variety of cancer types. In liver cancer, Zhao et al. revealed the downregulation of miR-148b in the liver cancer cell lines, such as HepG2, MHCC97H, and MHCC97L [31]. Moreover, Huang et al. found a relatively lower level of miR152 in HBV-related HCC tissues than adjacent noncancerous hepatic tissues [32]. In gastrointestinal cancers, Chen et al. detected a downexpression of miR-148a and miR-152 in cancer tissues and cell lines, and also suggested the low level of miR-148a and miR-152 was associated with large tumor size and advanced stage [17]. DNA methylation of genes encoding miR-148/152 family members was observed in various cancers, which may be considered as an important mechanism of miR-148/152 family implicated in the carcinogenesis. In gastric cancer, DNA hypermethylation of miR-148a was mediated through upregulated DNMT1. Interestingly, silencing of miR-148a may further promote the overexpression of DNMT1, leading to enhancement of DNA hypermethylation [33]. DNMT was inversely associated with miR-148a/152 expression. The studies of Lujambio et al. showed that silencing of tumor suppressive miRNAs by DNA methylation might facilitate human cancer metastasis [34]. MiR-148/152 family regulates other pathways as well. Zheng et al. noted the inverse association between miR-148a level and lymph node metastasis in gastric cancer. They also reported that the migration and invasion of gastric cancer cells was suppressed through targeting Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) by miR-148a [35]. Besides, Song et al. implied that miR-148b may act as a tumor suppressor in gastric cancer and colorectal cancer. They suggested that the suppression of tumor growth might be executed by targeting cholecystokinin-2 receptor (CCK2R) [18, 36].

In our study, some inherited limitation should be noted. The NSCLC patients were all recruited from Asian population. The results may not fully represent the equal predictive performance in other ethnical group. Second, the selection of internal was crucial for the validity of PCR results. Since there was no widely accepted and applicable internal control for normalization, we selected U6 small RNA as internal control due to its stable and constant expression in both cases and controls. Concerning the number of patients, it's still not large enough to fully represent the entire population of NSCLC

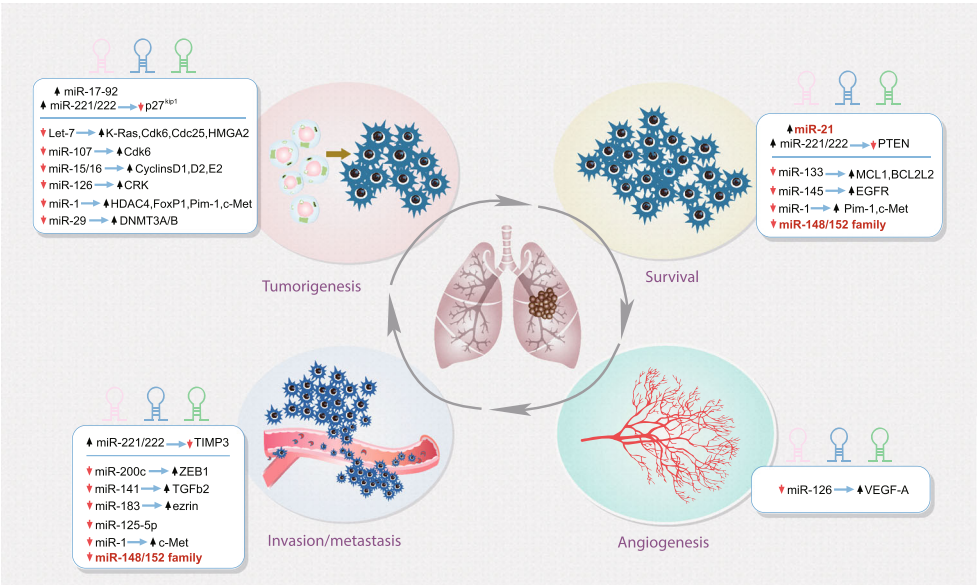

Fig. 2 Schematic diagram of miRNAs dysregulation in NSCLC

patients. It's recommended to conduct a large-scale investigation in further study to confirm our results.

In summary, our results showed an aberrant expression of four candidate miRNAs (miR-148a, miR-148b and miR-152, miR-21) in NSCLC patients and suggested the tumor suppressive role of miR-148/152 family and in contrast the oncogenic role of miR-21. They exhibited high predictive accuracy in NSCLC detection and have great potential to serve as novel non-invasive biomarkers. Further studies may be needed to validate our findings before its application in clinical settings.

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