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CLINICAL EVALUATION OF A PANEL OF CIRCULATING MICRORNAS IN NON-SMALL CELL LUNG CANCER

Laboratory Medicine

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ABSTRACT

Background: MicroRNAs (miRNAs) play critical role in the carcinogenesis of non-small cell lung cancer (NSCLC). Many serum miRNAs have been identified as biomarkers for NSCLC. In this study, we examined the expression of serum miRNAs (miR-145, miR-155, miR-182, miR-197, miR-205, and miR-210), and evaluated their diagnostic value in NSCLC. **Methods:** MicroRNAs were extracted from serum samples of 33 healthy volunteers, 29 patients with benign lung disease (BLD), and 55 NSCLC patients. The expression of miRNAs was detected by quantitative real-time PCR. **Results:** We found that the serum miR-155, miR-210, miR-197, and miR-182 levels were significantly increased in NSCLC, while the expression of miR-145 was substantially decreased in NSCLC. In addition, miR-155, miR-210, and miR-145 were significantly changed in all stages and subtypes. The expression of miR-182 was mainly increased in late stage, while miR-197 was mainly elevated in early stage. Moreover, miR-197 and miR-182 were mainly increased in squamous cell carcinoma (SCC). Finally, the receiver operating characteristic (ROC) curve analysis indicated that combination of miR-155, miR-210 and miR-145 can be used as an ideal serum biomarker panel in the diagnosis of NSCLC. **Conclusions:** These data suggest that combination of circulating miR-155, miR-210, and miR-145 can be further evaluated as novel potential biomarkers in NSCLC.

KEYWORDS

microRNA; non-small cell lung cancer; serum; diagnosis

INTRODUCTION

Lung cancer is one of the most prevalent cause of cancer related mortalities worldwide(1). Microscopically, lung cancer is classified as small cell and non-small cell lung cancer (NSCLC). Non-small cell lung cancer (NSCLC) accounts more than 80% of all lung cancer cases. The two main subgroups of non-small cell lung cancer (NSCLC) are adenocarcinoma and squamous cell carcinoma (SCC), with a remaining third class of carcinomas devoid of histological features of adeno- or squamous- differentiation, named large cell carcinoma (LCC)(2). Despite great advances in diagnostic and therapeutic modalities, the overall 5-year survival rate of NSCLC is still poor(3). This high mortality is due to the poor prognosis of the disease caused by a late disease presentation, tumor heterogeneities within histological subtypes, and the relatively limited understanding of tumor biology.

Accumulating evidences show that microRNAs (miRNAs) contribute to the carcinogenesis of many malignancies, including NSCLC(4). miRNAs are a novel class of small, endogenous, single stranded non-coding RNAs containing 18-25 nucleotides that play a crucial role in post transcriptional gene silencing through mRNA targeting. They bind to the 3′-untranslated region (3′-UTR) of target mRNAs to control target genes by inhibiting mRNA translation or degradation(5). miRNAs are predicted to regulate approximately 60% of all human genes and are involved in diverse biological processes including development, differentiation, metabolism, proliferation, cell cycle, and inflammation(6, 7).

Abnormal expressions of miRNAs have frequently been observed in various types of cancers(8). Numerous miRNAs have been deregulated in NSCLC and they act as oncogenes or tumor suppressors(9, 10). Several studies have detected the expression of microRNA in tumor tissue, cells and even in sputum of lung cancer patients. Nowadays, circulating miRNAs have been identified in serum/plasma in a remarkably stable form, and many serum miRNAs have been identified as biomarkers for various cancers, including NSCLC(11, 12). In this study, we selected a panel of miRNAs that have been reported to be altered in lung cancer and determined the circulating levels of those miRNAs for diagnosis of lung cancer.

MATERIALS AND METHODS

Study subjects and specimen preparation

A total of 55 lung cancer patients, 29 subjects with benign lung disease and 33 healthy controls were enrolled in our study from Tongji Hospital, Wuhan. The study involved 38 male and 17 female patients between the age of 40-80 years. All the patients were newly diagnosed and histologically confirmed with primary non-small cell lung cancer, not receiving any radiotherapy, chemotherapy or surgery and not having any co-existing illness. Among these NSCLC patients, 23 were squamous cell carcinoma and 32 were adenocarcinoma. The patients were at various clinical stages including 14 of stage I, II and III respectively and 13 of stage IV, as determined according to the International Association for the Study of Lung Cancer staging system. Age and sex matched patients with benign lung disease were included but none of them had previously been diagnosed with malignancy. In addition, a set of age and sex matched healthy volunteers, none had previously been diagnosed with any malignancies, or immune disorders or pulmonary diseases recently and who received physical examination at the same hospital were enrolled. Informed written consents were obtained from each subject, and this work confined to guidelines set forth by the Declaration of Helsinki and was approved by the ethics committee of the Tongji Medical College, Huazhong University of Science and Technology.

Five milliliters of peripheral blood was drawn from the patients and controls using standardized phlebotomy procedure. Serum was separated by immediate centrifugation at 3000 rpm for 10 min and transferred to a fresh tube and stored at – 80°C until RNA isolation. During the sample storage, repeated freeze-thawing was avoided to ensure the quality of the samples.

RNA extraction and Reverse Transcription

Total RNA containing miRNA was extracted from 100µl of serum using the QiaGen miRNeasy Mini Kit (QiaGen, Hilden, Germany) according to the manufacturer's instructions. The final elution volume was (14 µl) 100µl in water. The concentration and purity of sample RNA were determined by Thermo Scientific NanoDrop 2000 Spectrophotometer.

100 ng total RNA was reverse-transcribed in a final volume of 20µl RT reactions using miScript RT-Kit according to the manufacturer's protocol.

MiRNAs quantitative Real time PCR (qRT-PCR)

The expression of three reference genes (miR-16, SNORD68, and U6) as well as the expression of the miR-205, miR-155, miR-210, miR-145, miR-197, and miR-182 were determined by real-time PCR using miScript-System including miScript SYBR-Green PCR-Kit and miScript Primer Assay according to the manufacturer's protocol. All PCR reactions were performed in 96-well plates in duplicate and measured by Light Cycler 480 system (Roche, Mannheim, Germany). Mean values and standard deviations were calculated, and fold changes were determined using the $2^{-\triangle\triangle Ct}$ method.

Statistical analysis

Data were presented as mean ± standard deviation (SD) and analyzed using SPSS16.0. Differences between groups were examined by One-way ANOVA. To compare categorical data, Chi-Square Test was performed. The correlation between serum miRNAs and clinicopathological features was examined by Pearson Chi-Square Test. The ROC curve analysis was used to determine the diagnostic value of serum miRNAs in NSCLC. Binary Logistic Regression Analysis was used for combined ROC analysis. $P< 0.05$ was considered statistically significant.

RESULTS

Selection of the reference gene for serum miRNAs expression in lung cancer

Proper normalization is essential for accurate miRNA expression analysis, the use of reference gene as endogenous control is the most common method of normalization. In this study, three candidate reference genes (miR-16, SNORD68, and U6) were chosen for serum miRNAs expression in NSCLC patients and cancer-free controls. The expression of candidate reference genes were presented as in figure 1. The mean CT values of the genes ranged from 28.16 to 30.51. U6 exhibited a relatively low expression level, with a median CT of 30.67, whereas miR-16 was moderately abundant with a median CT of 28.96. SNORD68 was highly expressed in serum with a median CT of 28.30. In addition, miR-16 showed the least variation (CV of 3.2%), whereas SNORD68 (CV of 4.9%) was the most variable.

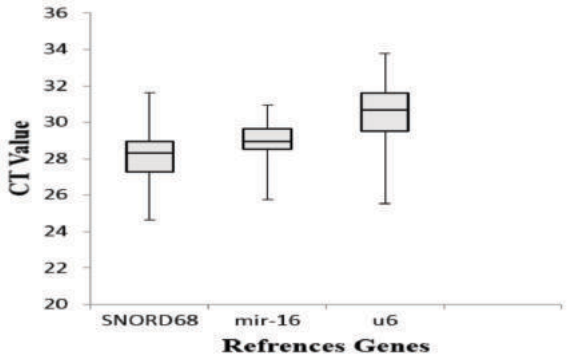

Figure 1: Boxplot of CT value of candidate Reference gene in serum sample

Generally, suitable reference gene for normalization should not demonstrate differential expression among different groups. In order to validate this, we evaluated the expression of candidate reference genes among different groups (healthy control, BLD and NSCLC patients) and found no evidence for differential expression of any of them (p>0.05) in this study (Table 1).

Table 1: CT value of candidate reference genes in different groups

Name	CT Min	CT Max	Mean CT±SD Healthy Control	Mean CT±SD BLD	Mean CT±SDLC Patients	P Value
miR-16	25.76	30.96	29.01±0.74	29.04±0.73	28.97±1.13	0.200
U6	25.53	33.78	30.64±1.58	30.57±.1.57	30.39±.1.37	0.727
SNORD 68	24.65	31.31	28.00±0.86	27.51±1.33	28.60±1.49	0.953

In order to evaluate the expression stability of these three candidate reference genes, we performed statistical analysis of their expression with four independent algorithmths, BestKeeper, NormFinder, the comparative delta-Ct method, and RefFinder(13-16). BestKeeper analyzed the variability by calculating the Ct set SD and coefficient of variance (CV). NormFinder used a model-based approach which took into account the intra- and inter-group variations of the candidate genes in addition to the overall expression variation to evaluate expression stability. The comparative delta-Ct method compared expression of gene pairs within each sample and ranked the stability according to the repeatability of the expression difference. In the above three programs, miR-16 was the most stable reference gene. Based on the rankings from BestKeeper, NormFinder, and the comparative delta-Ct method, RefFinder assigned an appropriate weight to each gene and calculated the geometric mean of their weights for the overall final ranking. This analysis confirmed that miR-16 was the most suitable reference gene for the calculation of serum miRNAs expression (Table 2). Therefore, the relative expression of serum miRNAs was normalized with miR-16 and analyzed in subsequent analysis.

Table 2: Expression stability of candidate reference genes

Rank	Gene Symbol	BestKeeper SD[±CP]	NormFinder (stability value)	Delta Ct (Mean SD)	Comprehensive (RefFinder)
1	miR-16	0.75	0.814	1.63	1
2	U6	1.10	1.366	1.79	1.86
3	SNORD68	1.17	1.457	1.83	2.71

Relative expression of serum miRNAs in NSCLC

There was no significant difference among healthy control subjects, BLD and NSCLC patients in the clinical characteristics, including gender, age, and smoking habit. The relative expression of serum miR-205, miR-155, miR-210, miR-145, miR-197, and miR-182 were determined by qRT-PCR. There was no significant difference in the expression of the above miRNAs between the healthy controls and BLD subjects. The results showed that expression levels of miR-155, miR-210, miR-197, and miR-182 were significantly increased in NSCLC by 3.70, 2.23, 1.56, and 1.72 folds respectively compared with the healthy control, while the expression of miR-145 was substantially decreased in NSCLC by 1.71 folds. At the same time, the expression of miR-205 was not remarkably changed (Figure. 2).

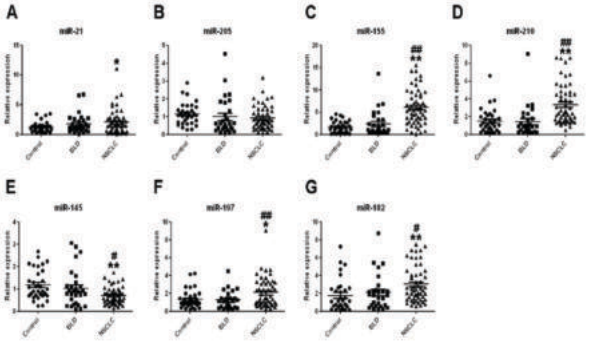

Figure 2: Expression of Serum miRNAs in NSCLC

Correlation between serum miRNAs and clinicopathological features

We further analyzed the correlation between the serum miRNAs expression levels and clinicopathological features of NSCLC patients. NSCLC patients were divided into two groups according to their clinical stage. I/II stages were grouped as early stage, and III/IV were grouped as late stage. We compared the expression of these miRNAs in different stages of NSCLC patients, the healthy controls and BLD subjects. The data showed that miR-155 and miR-210 were significantly increased in both early and late stages compared to the healthy controls or BLD subjects, while miR-145 was substantially decreased in both stages. Interestingly, miR-197 was only remarkably increased in early stage without significant change in late stage, while miR-182 was only significantly increased in late stage NSCLC patients. MiR-205 was not significantly changed in either stage of NSCLC patients compared to the healthy controls or BLD subjects (Figure 3)

Furthermore, we examined the expression of these miRNAs in the subtypes of NSCLC, Squamous cell carcinoma (SCC) and adenocarcinoma (ADC). We found that miR-155 and miR-210 were significantly increased in both SCC and ADC patients compared with the healthy controls or BLD subjects, while miR-145 was substantially decreased in both subtypes. MiR-205 was not significantly changed in either subtypes of NSCLC patients compared with the healthy control

subjects. Interestingly, miR-197 and miR-182 were only remarkably increased in SCC patients without significant change in ADC patients, suggesting that the expression of miR-197 and miR-182 may be associated with SCC rather than ADC (Figure 4).

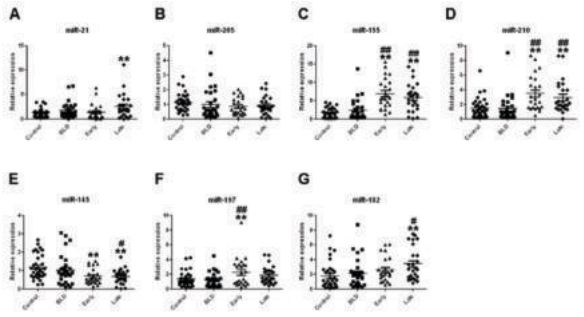

Figure 3: Expression of Serum miRNAs in Different Stages of NSCLC Patients.

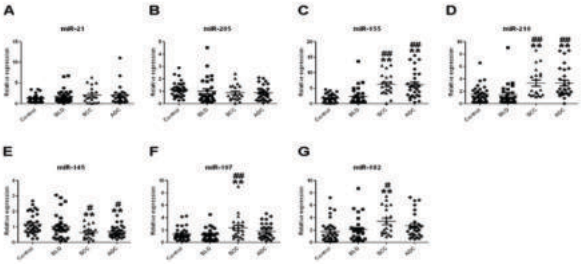

Figure 4: Expression of serum miRNAs in different subtypes of NSCLC patients.

Diagnostic value evaluation of serum miRNAs in NSCLC by ROC curve

The ROC curve analysis was used to explore the diagnostic value of the above miRNAs in NSCLC and cancer-free controls. The Area Under the Curve of these miRNAs were shown in (Table 3).

Table 3: Area Under the Curve

miRNAs	AUC	P value	95% CI		Positive	Optimal sensitivity	Optimal specificity
			Lower Bound	Upper Bound			
miR-21	0.554	0.331	0.442	0.665	1.474	0.529	0.782
miR-205	0.457	0.430	0.350	0.564	0.271	0.904	0.115
miR-155	0.841	0.000	0.768	0.915	3.306	0.745	0.836
miR-210	0.804	0.000	0.726	0.883	1.259	0.926	0.607
miR-145	0.669	0.002	0.569	0.768	0.688	0.758	0.558
miR-197	0.652	0.005	0.552	0.753	2.122	0.455	0.833
miR-182	0.694	0.000	0.599	0.788	0.790	0.944	0.387
Combination	0.925	0.000	0.877	0.973	0.486	0.824	0.885

Combination : miR-155, miR-210, and miR-145.

Results of ROC curve suggested that the diagnostic value of these serum miRNAs were miR-155>miR-210>miR-182>miR-145>miR-197, yielding AUC of 0.841, 0.804, 0.694, 0.669, and 0.652 respectively (Figure 5).

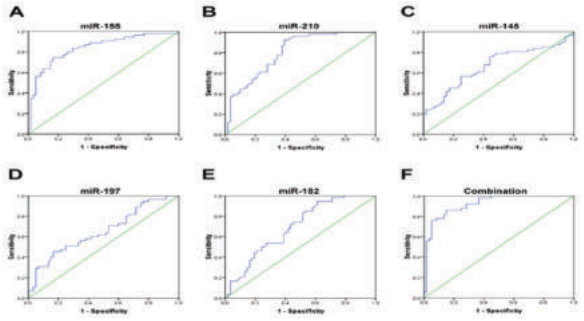

Figure 5: The receiver operating characteristics (ROC) curve analysis.

The combined use of different miRNAs were tested for the diagnostic value through Binary Logistic Regression Analysis and the top 4 combinations were miR-155/miR-210/miR-145, miR-155/miR-210/miR-145/miR-182, miR-155/miR-210/ miR-145/miR-197, and miR-155/miR-210/ miR-145/miR-197/miR-182 with AUC of 0.925, 0.925, 0.924 and 0.922 respectively. No significant difference was found among the top 4 combinations by Z test.

DISCUSSION

A number of genetic and epigenetic factors have been involved in the development and progression of NSCLC, and increasing evidences reveal the role of miRNAs in the carcinogenesis of NSCLC(17-19). Biogenesis of miRNAs begins at nucleus then they are transported to cytoplasm where further processing leads to maturation. Previously, cells and tissues were thought to be the only possible source of miRNAs and most of the studies had examined miRNA expression levels taking cells or tissues as samples. But recently, it was found that miRNAs can be released into the circulation and exist in a remarkably stable form due to resistance to RNase A digestion. Furthermore, various tumors including lung cancer were shown to leave specific miRNA fingerprints in the blood of patients, suggesting that circulating miRNAs could serve as non-invasive biomarkers for the diagnosis of these diseases. In addition, the valuable characteristics of circulating miRNAs such as high stability, noninvasiveness, reproducibility, low cost and consistency among individuals of same species have garnered our attention to consider it as biomarker(20, 21). In this study, we analyzed a panel of serum miRNAs in healthy controls, BLD patients and NSCLC patients with an attempt to reveal the diagnostic signature of unique miRNA in NSCLC.

Proper normalization of RT-qPCR data is crucial for accurate quantification of miRNAs as normalization to an unsuitable reference gene has been shown to lead to biased results(22). In this study, we applied three widely used reference genes (U6, miR-16 and SNORD68) to determine the most stably expressed reference gene. Among these, U6 and miR-16 were frequently used as reference genes in previous studies and expression levels of miRNAs from different materials (including cell line, tissue, plasma/serum, sputum, etc) were found to be normalized with them. Meanwhile SNORD68 was easily available commercially and recommended as stable reference gene. miR-16 was selected as the reference gene in this study for further analysis.

miR-155 is one of the microRNAs that is most consistently involved in various malignant diseases including lung cancer and first miRNAs to be associated with NSCLC development (23). Significantly high serum concentrations of miR-155 was demonstrated in NSCLC when compared with cancer-free controls. MiR-155 individually reached the highest AUC at 0.841, showing moderate to high diagnostic accuracy for this disease. Compared with BLD and healthy controls, miR-210 was also significantly overexpressed in both early and late stage NSCLC patients, yielding the second highest AUC of 0.804.

MiR-145 in our study was drastically under expressed in patients compared with both BLD and healthy controls irrespective of stages and subtypes of lung cancer. In the previous study, it was demonstrated that overexpression of miR-145 markedly inhibited proliferation of human lung adenocarcinoma cell lines, which partly by targeting c-Myc(24). During lung carcinogenesis, the loss of miR-145 may provide a selective growth advantage, suggesting that miR-145 was a potential tumor suppressor in NSCLC(25). Our study indicated that serum miR-145 might act as a potential tumor marker of NSCLC but this individual miR showed poor diagnostic efficiency with an AUC value less than 0.7.

Results of miRNAs expression may be helpful for histological diagnosis of NSCLC. Our results demonstrated serum expression of miR-182 was only significantly increased in SCC, suggesting it as a potential biomarker to distinguish SCC from ADC. The possible mechanism of this differentiation might be due to the ability of miR-182 to suppress cell proliferation by down regulating RAS A1, a gene involved in cell apoptosis in SCC(26).

According to previous researches, miR-197 may play inverse role in different kinds of tumors. Wang et al. found miR-197 expression was downregulated in astrocytoma, oral squamous cell carcinoma and multiple myeloma, and downregulation of miR-197 was associated with poor prognosis in esophageal cancer(27). However, Fiori et al.and

Du et al. reported miR-197 was upregulated in lung cancer and acted as oncogenic miRNAs by inhibiting tumor suppressor genes (28, 29). In this study, we found circulating miR-197 was drastically elevated in SCC patients but not in ADC. In addition, upregulation of serum miR-197 occurred mainly in patients at early stage. The mechanism that elevation of miR-197 was dependent on histological type and tumor stage in NSCLC requires further study and may provide clues to clarify functional role of miR-197 in tumorigenesis.

Moreover, limited diagnostic values were found in ROC curve analysis for miR-182 and miR-197, together with miR-205 in this study compared with a few of previous studies(30-32). This could be partly explained by the fact that in this study both BLD patients and healthy controls were included in the cancer-free controls. some benign lung diseases also displayed aberrant miRNA expression according to recent researches. For example, the serum levels of miR-155 and miR-197 were significantly elevated in the pneumonia patients whereas miR-182 and miR-197 levels were increased in patients with TB(30). Besides, the inconsistency may also arise from the differences in study design, race, sample size, and also methodology.

Combination of several serum miRNAs expression have been identified to be reproducible and consistent among individuals. The ROC curve analysis in our study showed that the combination of miR-155, miR-210, and miR-145 yielded the AUC of 0.925 ($P<0.0001$). Meantime, addition of miR-197 or miR-182 did not increase the diagnostic value of this combination. The optimal sensitivity and specificity were 82% and 89% respectively, suggesting that the combination of miR-155, miR-210 and miR-145 can serve as a potential noninvasive biomarker panel for the diagnosis of NSCLC.

In summary, our study supports the value of serum miRNAs as noninvasive biomarkers for the diagnosis of NSCLC. Further research to elucidate the functional roles of these miRNAs will expand our knowledge of these novel biomarkers in carcinogenesis of NSCLC. Confirmation of aberrant expression of these miRNAs in a large prospective NSCLC study will provide support for its application to NSCLC detection. These data provide a scientific experimental basis for the further studies about their clinical value.

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Conflict of interest:
The authors have declared that no potential conflict of interests exist.

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