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Original Article

Evaluation of miR-34a, CEA, CA125, and ProGRP combined detection effect in NSCLC diagnosis and prognosis

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Abstract: Non-small cell lung cancer (NSCLC) is responsible for more than 80% of lung cancer. MiR-34a plays a tumor suppressor role in lung cancer. CEA, CA125, and ProGRP are common serum biomarkers in the diagnosis of NSCLC. This study intends to explore the role of serum markers combined with miR-34a detection in NSCLC diagnosis and prognosis. Serum was extracted from NSCLC patients, a high-risk group, and from healthy volunteers. Real time PCR was used to test miR-34a expression. ELISA was used to detect CEA, CA125, and ProGRP levels. NSCLC specificity and sensitivity were combined and analyzed. MiR-34a expression was significantly decreased in NSCLC patients and the high-risk group compared with controls ($P < 0.05$). It was reduced in NSCLC patients compared with the high-risk group following TNM stage increase ($P < 0.05$). CEA and CA125 were clearly overexpressed in NSCLC patients compared with the high-risk and control group ($P < 0.05$). No statistical difference of ProGRP was observed between each group. Combined detection revealed that the four marker combination had the highest sensitivity (97.2%). MiR-34a, CEA, and CA 125 showed the highest sensitivity (90.1%) in three marker detection. CEA and CA125 presented highest sensitivity (71.6%) among two marker detection. MiR-34a combined with CEA, CA125, and ProGRP can elevate lung cancer detection sensitivity, which is important for NSCLC screening, diagnosis, and prognosis.

Keywords: Lung cancer, serum biomarker, miR-34a, prognosis

Introduction

Lung cancer is a kind of malignant tumor in the respiratory system, which can be divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for more than 80% of lung cancer [1]. NSCLC includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma, all of which show lower malignancy compared with SCLC. Lung cancer clinical symptoms are different according to location, tumor size, and metastasis range [2]. Most patients are often diagnosed in advanced stage due to hidden symptoms in early stages, high malignancy, and rapid progression. Therefore, lung cancer presents as one of the leading morbidity and mortality causes worldwide [3, 4]. In recent years, lung cancer has shown increasing mortality, mortality, and a younger trend following life rhythm changes and environmental pollution [5, 6]. Lung cancer occurrence and development is a multi-factor, multi-step, polygenic, and multi-level complex process. Most lung cancer patients show poor prognosis because of early diagnosis method deficiency and poor therapeutic effect for advanced lung cancer [7, 8]. Early diagnosis method mainly includes CT, X-ray, and sputum cytology examination. However, due to the low sensitivity and specificity, missed diagnosis and misdiagnosis still exist [9]. Thus, early detection, diagnosis, and treatment are important means for lung cancer prevention and to improve patient's quality of life and survival.

MicroRNA (miRNA) is a kind of small non-coding RNA with regulatory functions that are widely preserved in animals and plants. MiRNAs can negatively regulate gene expression by com-

Table 1. Primer sequence

Gene	Forward 5'-3'	Reverse 5'-3'
GADPH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
Mir-34a	ACAGTCAGCAAGTCTCAATGG	GCTTCGCGACATCTCCGTA

plete or incomplete binding with target genes. Complete matching leads to RNA degradation by RISC protein complex in the cytoplasm, while incomplete matching may inhibit the protein translation process [10, 11]. MiRNA participates in controlling cell proliferation, apoptosis, signal transduction, cell differentiation, hormone secretion, fat metabolism, and maintains embryonic stem cell potential; so as to regulate body development and enhance the bodies adaptive capacity in the environment [12, 13]. A current study found that miRNA is closely related to tumor occurrence and development [14]. MiR-34a plays a tumor suppressor role in lung cancer [15]. CEA, CA125, and ProGRP are common serum markers in NSCLC diagnosis. However, whether miR-34a can diagnose NSCLC and help determine the prognosis by combining with CEA, CA125, and ProGRP detection levels is still unknown and of major interest.

Materials and methods

Patient selection

A total of 78 NSCLC patients diagnosed by histopathology between June 2014 and June 2015 were enrolled in the Affiliated First Hospital of Quanzhou, Fujian Medical University. All patients received surgical treatment, including 41 males and 37 females with mean age at 62.2 ± 7.9 (51-77) years old. There were 19 cases with patients in stage I, 25 patients in stage II, 21 patients in stage III, and 13 patients in stage IV. There were 35 cases of squamous carcinoma, 27 cases of adenocarcinoma, and 16 cases of large cell carcinoma. Inclusion criteria: All patients received surgery with no chemotherapy or radiotherapy before surgery. All patients had signed informed consent. Exclusion criteria: recurrent lung cancer; received surgery with chemotherapy, or radiotherapy beforehand; combined with other disease at admission, such as infectious disease, malignant tumor, severe diabetes, severe liver and kidney disease, pulmonary fibrosis, bone metabolic disease, immune disease, and malignant tumor complications. Another 62 patients with high risk lung cancer factors (age > 50 years old, smoking history, lung cancer family history) were selected, including 37 males and 25 females with mean age at 62.4 ± 7.2 (52-75) years old as the high-risk group. A total of 60 healthy volunteers were selected as controls, including 31 males and 29 females with mean age at 63.8 ± 6.7 (51-77) years old. No statistical difference about general health information was observed among the three groups.

The study protocol was approved by the Research Ethics Committee of the Affiliated First Hospital of Quanzhou, Fujian Medical University, and all patients gave their informed consent before study commencement.

Main reagents and instruments

CEA, CA125, and ProGRP ELISA detection kits were bought from R&D (USA). Labsystem Version 1.3.1 microplate reader was acquired from Bio-rad (USA). RNA extraction kit and reverse transcription kit were purchased from RD (USA). Other common reagents were from Sangon (China). Real time PCR reagents were purchased from Thermo Fisher. Real time PCR amplifier was from ABI (USA). DNA amplifier was from PE Gene Amp PCR System 2400 (USA).

Methods

Specimen collection: A total of 2 ml fasting blood was collected from each group using negative pressure acquisition method. The blood was placed at room temperature for 30 min and then centrifuged at 4°C and 3600 rpm for 10 min. The supernatant was stored at -20°C.

Real-time PCR: Total RNA was extracted using Trizol and reverse transcription was used to generate cDNA using primers (**Table 1**). Real time PCR was used to detect target gene expression. The process consisted 92°C for 1 min, followed by 35 cycles of 90°C for 30 s, 58°C for 50 s, and 72°C for 35 s. CT number was calculated according to GAPDH. Gene relative expression level was analyzed by $2^{-\Delta Ct}$ method.

ELISA: Serum was collected to detect CEA, CA125, and ProGRP expression using ELISA. 50 μl diluted standard substance was added to a 96-well plate to prepare the standard curve. 50 μl of sample was added to each well with

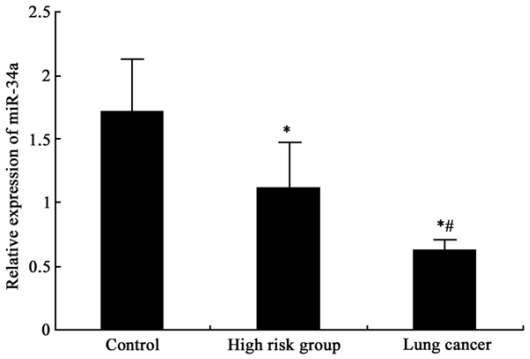

Figure 1. MiR-34a expression changes in each group. $^{*}P < 0.05$, compared with control; $^{\#}P < 0.05$, compared with high risk group.

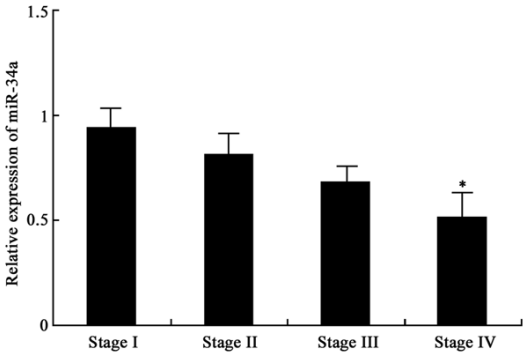

Figure 2. MiR-34a expression changes in different TNM stages. $^{*}P < 0.05$, compared with TNM stage I.

three replicates. After washing the well 5 times, 50 μl enzyme reagent was added and kept at 37°C for 30 min. After washing the plate 5 times, 50 μl of reagent A and 50 μl of reagent B were added at 37°C for 10 min. The reaction was terminated by 50 μl stop buffer, and the plate was read at 490 nm to obtain the OD value. The linear regression equation of standard curve was calculated using standard substance concentration and OD value. Sample concentration was calculated based on the regression equation.

Sensitivity and specificity analysis

Single or combined serum tumor marker detection sensitivity and specificity of NSCLC was analyzed. Sensitivity was the ratio of true positive number with true positive number and false negative number. Specificity was the ratio of true negative number with true negative number and false positive number.

Statistical analysis

SPSS 16.0 software was applied for data analysis. Measurement data was presented as mean ± standard deviation ($\bar{x} \pm SD$) and compared by one-way ANOVA or LSD test. Enumeration data was presented as percentage (%) and compared by chi-square test. $P < 0.05$ was considered as statistical significance.

Results

Decreased miR-34a expression in NSCLC

Real time PCR was used to test miR-34a expression changes in NSCLC patients, high-risk, and control group. The results showed that miR-34a expression significantly decreased in NSCLC patients and the high-risk group compared with controls ($P < 0.05$). It also reduced in the high-risk group compared with control group ($P < 0.05$) (**Figure 1**).

Association of miR-34a expression with TNM stages

Real time PCR was used to detect miR-34a expression changes in NSCLC patients at different TNM stages. It was revealed that miR-34a level clearly declined following TNM upstage in NSCLC patients. MiR-34a expression in stage IV was markedly lower than that in stage I ($P < 0.05$). However, miR-34a expression did not have a statistical difference among different pathological types, such as squamous cell carcinoma, adenocarcinoma, and large cell carcinoma (**Figure 2**).

No association of miR-34a expression with pathological lung cancer types

Real time PCR was performed to analyze miR-34a expression in different pathological lung cancer types. The results demonstrated that miR-34a expression showed no significant difference among squamous cell carcinoma, adenocarcinoma, and large cell carcinoma (**Figure 3**).

Increased CEA and CA125 levels in NSCLC

CEA, CA125, and ProGRP levels in NSCLC patients, the high-risk group, and healthy volunteers were tested. The results showed that CEA was clearly elevated in the NSCLC group compared with controls ($P < 0.05$). Though it exceeded the normal range, CEA level in the high-risk group did not have statistical significance

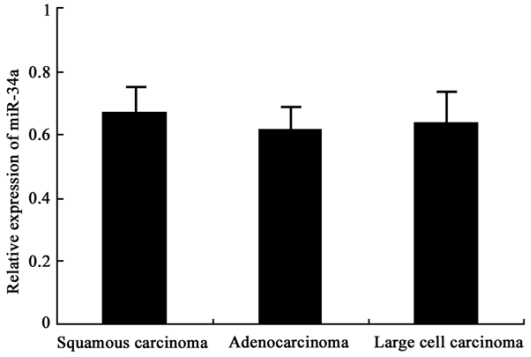

Figure 3. MiR-34a expression changes in different pathological lung cancer types.

Table 2. CEA, CA124, and ProGRP levels comparison between each group

	CEA (ng/ml)	CA125 (U/ml)	ProGRP (ng/ml)
Control	2.1 ± 0.5	21.3 ± 4.1	3.8 ± 1.2
High risk group	3.4 ± 0.2	23.6 ± 2.7	4.3 ± 1.7
NSCLC group	5.7 ± 0.9*,#	37.5 ± 6.7*,#	5.1 ± 0.9

*$P < 0.05$, compared with control; #$P < 0.05$, compared with the high-risk group.

compared with controls. CA125 was enhanced in the NSCLC group compared with the high-risk and control group ($P < 0.05$), while its level in the high-risk and control group was similar (**Table 2**). Different from the others, though its level increased in the NSCLC group, ProGRP showed no obvious difference between each group (**Table 2**).

High sensitivity and specificity of single serum marker detection in NSCLC

Four types of serum tumor marker expression was analyzed to determine the sensitivity and specificity. It was found that CA125 showed the highest sensitivity in NSCLC (65.1%); followed by CEA (51.1%) and miR-34a (58.3%). ProGRP showed the lowest sensitivity (21.2%). CEA presented the highest specificity (85.5%) among all markers, whereas ProGRP was the lowest (78.3%). No statistical difference was observed among each marker (**Table 3**).

Increased sensitivity and specificity of combined serum marker detection in NSCLC

Four markers were combined and analyzed to determine the combined sensitivity and specificity in NSCLC. The results demonstrated that the four markers combined had the highest sensitivity (97.2%). MiR-34a, CEA, and CA 125 showed the highest sensitivity (90.1%) in the three markers detection. CEA and CA125 presented the highest sensitivity (71.6%) among the two markers detection. On the specificity aspect, all four markers combined showed the highest detection value (82.6%), while two markers combined presented the lowest detection (79.7%). No statistical difference in specificity was observed between each combination (**Table 4**).

Table 3. Sensitivity and specificity of single serum marker detection in NSCLC (%)

Marker	Sensitivity	Specificity
CEA	51.1	85.5
CA125	65.1	82.1
ProGRP	21.2	78.3
miR-34a	58.3	80.4

ROC curve analysis of each marker in the diagnosis of NSCLC

We adopted the ROC curve to analyze the diagnostic value of each marker in NSCLC. The area under the curve of CEA, CA125, miR-34a and ProGRP were 0.752, 0.845, 0.673 and 0.812, respectively. The area under the curve of CEA+CA125, CEA+ProGRP, CEA+Mir-34a, CEA+CA125+ProGRP, CEA+CA125+miR-34a, CA125+ProGRP+miR-34a, CEA+CA125+ProGRP+miR-34a were 0.845, 0.752, 0.812, 0.862, 0.862, 0.845, 0.862, respectively (**Figure 4**).

Discussion

Lung cancer is the most common and easily recurrent respiratory tract malignant tumor type around the world. NSCLC is the highest majority type, with poor prognosis. Even though a large advance has been reached in surgery treatment combined with radiotherapy and chemotherapy, its postoperative recurrence rate is still high. The average survival period is short [16]. In recent years, following improved understanding of lung cancer biology and study of its pathogenesis development, lung cancer diagnosis and treatment had been improved. However, there is still a lack of specific markers for lung cancer prevention, early diagnosis, and treatment. Lung cancer is still a global problem,

Table 4. Sensitivity and specificity of combined serum marker detection in NSCLC (%)

Tumor marker	Sensitivity	Specificity
CEA+CA125	71.6	80.2
CEA+ProGRP	59.1	79.7
CEA+Mir-34a	68.2	80.1
CEA+CA125+ProGRP	82.2	81.5
CEA+CA125+miR-34a	90.1	81.7
CA125+ProGRP+miR-34a	81.7	80.7
CEA+CA125+ProGRP+miR-34a	97.2	82.6

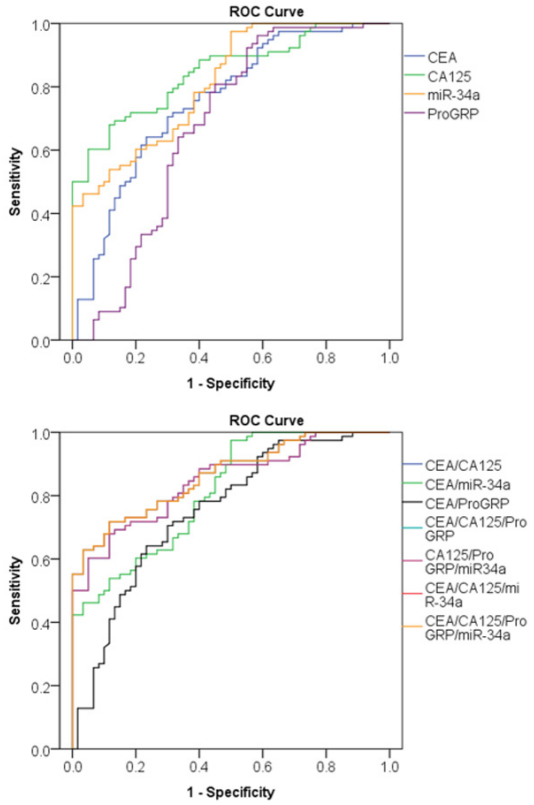

Figure 4. ROC curve analysis of each marker in the diagnosis of NSCLC.

needing to be urgently solved; with the early detection of lung cancer, improving treatment effects, improving prognosis, lowering morbidity and mortality rates, and elevating the survival rate [17]. Tumor markers have been widely investigated in molecular biology, and some are used in the clinic following proteomics and gene chip technology development. Some of the serum markers have been widely used in lung cancer clinical diagnosis, curative effect evaluation, and recurrence monitoring [18].

Detecting serum markers has positive clinical significance for lung cancer screening and early diagnosis. At present, CEA, CA125, and ProGRP are commonly used as lung cancer serum tumor markers. Carcinoembryonic antigen (CEA) contains human embryonic antigen specificity determinant and overexpresses in a wide variety of tumors that lack specificity. CA125 has a certain value for lung cancer auxiliary diagnosis and differential diagnosis. ProGRP is mainly used for SCLC and NSCLC identification, and it is also used for metastatic lung cancer detection [19, 20]. All of these three markers showed low specificity and sensitivity for lung cancer in single use. Thus, it is needed to find serum markers with higher specificity and sensitivity for lung cancer diagnosis. This study confirmed that CA125 had the highest sensitivity of the three, but only at 65.1%. Furthermore, the three markers combined failed to elevate the sensitivity significantly (82.2%). MiRNAs regulate genes expression at the post-transcriptional level. Circulating miRNAs can be used as biomarkers of disease, though the expression and mechanisms have not been fully elucidated. Studies showed that micro-vesicles can actively secrete miRNAs. *In vitro* experiments revealed that apoptotic bodies in endothelial cells can secrete miRNAs in serum-free medium. MiRNAs finish their integration in a microvesicle, apoptotic body, or exosome and then are secreted to the recipient cells to form miRNA complexes, which guarantee that RNAase cannot effectively degrade the circulating miRNAs. Circulating miRNA levels can reflect tumor progression and even evaluate the tumor size [21]. MiR-34a is confirmed to be a tumor marker that plays a role in lung cancer occurrence and development [15, 22]. This study confirmed that miR-34a expression was significantly decreased in lung cancer patients and in the high-risk group. Its level was clearly lower in lung cancer patients compared with the high-risk group, and significantly decreased following TNM upstage. Moreover, it showed no correlation with pathologic classification, suggesting that serum miR-34a could be treated as a new type of lung cancer marker. Our study further proved that although the sensitivity and specificity of miR-34a detection alone, in lung cancer was not high, the detection of the four serum markers combined presented the highest sensitivity. Following miR-34a, CEA and CA125 combined presented the highest sensitivity (71.6%) among the two markers detection. On the specificity aspect, detection of all four markers combined showed the highest

value, while detection of only two markers combined presented the lowest. No statistical difference in specificity was observed between each combination. This confirmed that miR-34a combined with CEA, CA125, and ProGRP contributed to accurate NSCLC diagnosis.

In brief, miR-34a combined with CEA, CA125, and ProGRP can elevate lung cancer detection sensitivity, which is important for NSCLC screening, diagnosis, and prognosis.

Disclosure of conflict of interest

None.

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