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Original Article

The aberrant expression of miR-485 in exhaled breath condensate among patients with non-small cell lung cancer and its clinical significance

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Abstract: Lung cancer is one of the most publicly known reasons of tumor-related mortality worldwide. It is especially important to search for effective methods of early diagnosis. In our study, real-time quantitative polymerase chain reaction (qRT-PCR) was used to detect the relative expression of miR-485 in serum and exhaled breath condensate (EBC) specimens from 72 patients with non-small cell lung cancer (NSCLC), also tissues and adjacent tissues were collected from 30 patients, and serum and EBC specimens were collected from 69 healthy subjects. Results show that the relative expression of miR-485 in serum, EBC and tissues of NSCLC patients were extremely downregulated compared to that of the controls. The relative expression miR-485 in stage III-IV NSCLC was obviously downregulated than that in stage I-II. The level of EBC miR-485 was related to lymph node metastasis and distant metastasis, but not to age, gender, smoking history nor the pathological type. The expression of serum miR-485 in NSCLC patients was related to platelet-lymphocyte ratio (PLR) and CEA. Correlation analysis was used to assess the relationship between miR-485 in three different types of samples. Finally, through receiver operating characteristics (ROC) curve and Z test, it was revealed that the detection of miR-485 in EBC had an efficient diagnostic value. To sum up, this study shows that the detection of miR-485 in EBC is feasible with great advantages, and miR-485 may be a promising biomarker in the diagnosis and evaluation of NSCLC.

Keywords: Non-small cell lung cancer, exhaled breath condensate, microRNA485

Introduction

Lung cancer is one the most generally acknowledged malignant tumors worldwide, endangering human health all of the time [1]. Although the rapid development of new treatment methods has made significant progress in recent years, about 70% of patients have developed distant metastasis by the time they are diagnosed. The overall survival rate of lung cancer in the United States is still only about 19% [2]. Therefore, it is important to focus on early screening, which is expected to improve the survival rate and improve the prognosis of lung cancer.

Exhaled breath condensates (EBC) is regarded as a convenient, safe and non-invasive method of collecting body fluid samples, and it can be easily and repeatedly sampled [3]. The compliance of patients is also well controlled. Therefore, its clinical value has attracted widespread attention. EBC contains thousands of volatile and non-volatile compounds [4]. With the maturation of detection technology, the types of media detected in EBC continue to increase, including various microRNAs (miRNA) [5-7].

miRNAs are a type of endogenous non-coding single-stranded RNA with a length of about 18 to 25 nucleotides and are widely found in eukaryotes [8]. According to the miRNA database, the human genome contains a total of 1917 precursor miRNAs and 2654 mature miRNAs [9]. Several studies have shown that miRNAs are dysregulated in lung cancer [10, 11]. miR-485 is located in chromosome 14q32.31,

Table 1. Characteristics of the study participants

Characteristic	NSCLC (n = 72)	Control (n = 69)	*P* value
Age (years)	64.540±9.307	62.800±7.397	0.221
Gender			
Male	39	37	0.948
Female	33	32	
Smoking			
Yes	38	35	0.807
No	34	34	
Histological type			
Adenocarcinoma	48	-	
Squamous cell carcinoma	24	-	
Lymph node metastasis			
Yes	30	-	
No	42	-	
Distant metastasis			
Yes	20	-	
No	52	-	
TNM stage			
I-II	38	-	
III-IV	34	-	

and is involved in regulating the occurrence and development of liver cancer, bile duct cancer, gastric cancer, bladder cancer, breast cancer and ovarian cancer, and it is also of great significance for further research on tumor drug resistance [12-18]. Mozzoni et al. detected abnormal expression of miRNA in EBC among patients with non-small cell lung cancer (NSCLC) for the first time, guiding a large number of scholars into the new research field of EBC and lung cancer [19]. However, miR-485 has not been detected in EBC of lung cancer patients so far. The purpose of our study is to investigate the abnormal level of miR-485 in NSCLC and its clinical significance.

Materials and methods

Study subjects

In total, 72 patients pathologically diagnosed with NSCLC in the Second Affiliated Hospital of Nantong University from November 2017 to August 2019, including 39 males and 33 females, were enrolled as the NSCLC group. The inclusion criteria were as follows: (a) all patients were diagnosed with NSCLC by pathological examination; (b) all subjects had complete clinical data. The patients were excluded if they had the following situation: (a) radiotherapy, chemotherapy, targeted therapy or immunotherapy had been performed before specimen collection; (b) chronic lung diseases; and (c) past history of heart, brain, liver, kidney, and other important organ diseases and malignant tumors. In accordance with the International Anti-Cancer Alliance 8th edition lung cancer TNM standard staging, there were 38 cases of stages I-II and 34 cases of stages III-IV NSCLC that were detected. We collected serum and EBC specimens of 72 patients, and collected paired tissue specimens of 30 patients. No anti-tumor treatment was administered to any of the patients before surgery. During the same period, 69 healthy subjects, including 37 males and 32 females, were set up as the control group. We collected serum and EBC specimens of the controls. No history of malignant tumor, major organ disease, and abnormal chest imaging were noted. There is no significant difference between the two groups in terms of age, gender, or smoking history (**Table 1**). Written informed consent was obtained from all the subjects upon approval of the study by the Ethics Committee of the Second Affiliated Hospital of Nantong University.

Samples collection

We collected fresh tumor tissue specimens and adjacent tissues within 30 minutes after the operation. Quickly we froze the samples in liquid nitrogen and store them at -70°C. EBC samples were collected using an EcoScreen condenser (Erich Jaeger GmbH, Hoechberg, Germany). Subjects were asked to bite and collect their mouthpieces after mouth washing. We were aware of air leaks and told the patients to breathe calmly for about 20 min. The condensation collection tube was removed, and we transfered 2-4 ml of the collected liquid to the RNase-Free centrifuge tube, and stored it at -70°C. All subjects gave 5 mL venous blood on an empty stomach in the early morn-

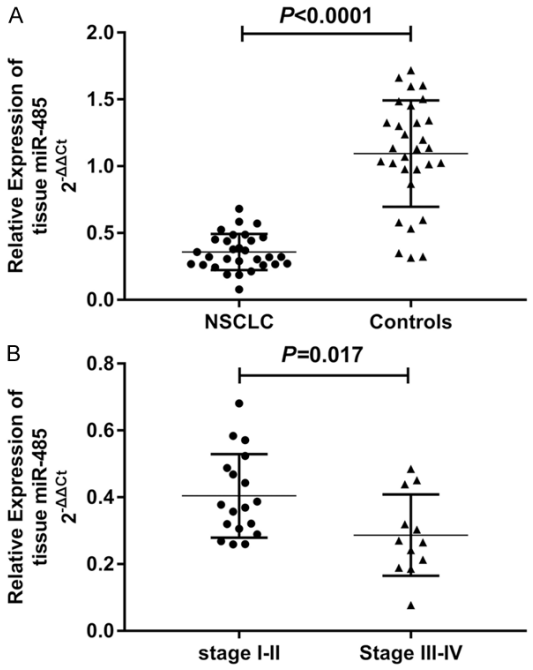

Figure 1. A. Comparison of miR-485 expression in lung cancer tissues and adjacent tissues of 30 NSCLC patients; B. Comparison of tissue miR-485 expression of patients at stage I-II and stage III-IV.

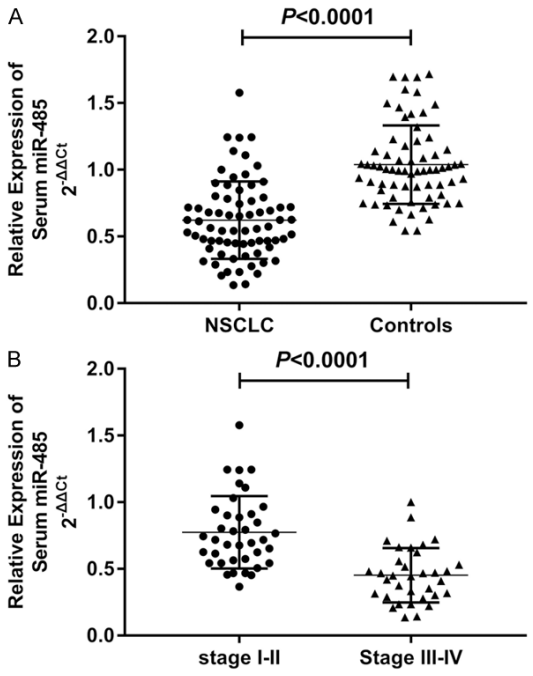

Figure 2. A. Comparison of serum miR-485 expression of 72 NSCLC patients and 69 healthy controls; B. Comparison of serum miR-485 expression of patients at stage I-II and stage III-IV.

ing. After centrifugation at 3500 r/min for 5 min, the serum was separated and stored at

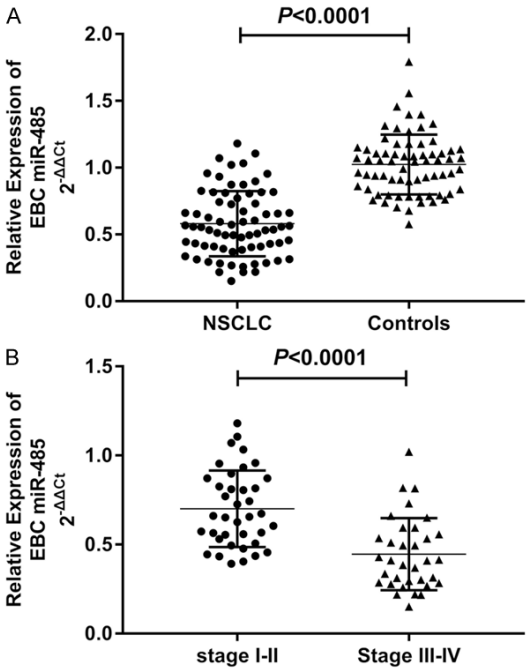

Figure 3. A. Comparison of EBC miR-485 expression of 72 NSCLC patients and 69 healthy controls; B. Comparison of EBC miR-485 expression of patients at stage I-II and stage III-IV.

-70°C. Leukocytes, neutrophils, lymphocytes, platelets, carcinoembryonic antigen (CEA), cytokeratin 19 fragments (CYFRA21-1) and squamous cell antigen (SCC) of the NSCLC group were obtained from the the clinical laboratory results.

RNA isolation and quantification reverse transcription-polymerase chain reaction (qRT-PCR)

A total of 200 μl serum miRNA was extracted using a miRNA extraction kit (miRcute Serum miRNA Isolation kit, Tiangen Biotech Co., Ltd). The purity (ratio of absorbance at 260 and 280 nm) and concentration of miRNAs were analyzed using the OneDrop™ OD-1000 spectrophotometer system. Then, 30 μl total RNA was obtained using a reverse transcription kit to produce cDNA (miRcute Plus miRNA First-Strand cDNA Synthesis kit, Tiangen Biotech Co., Ltd). miR-485 expression level was detected via qRT-PCR (miRcute Plus miRNA qPCR kit, Tiangen Biotech Co., Ltd). The PCR conditions included one step at 95°C for 15 min, followed by 40 cycles at 94°C for 20 s and 64°C for 34 s. Each group of samples was subjected to the same process thrice. The forward primer sequences of has-miR-485, cel-miR-39 and U6 were synthesized and purchased from Tiangen Biotech Co., Ltd. U6 was

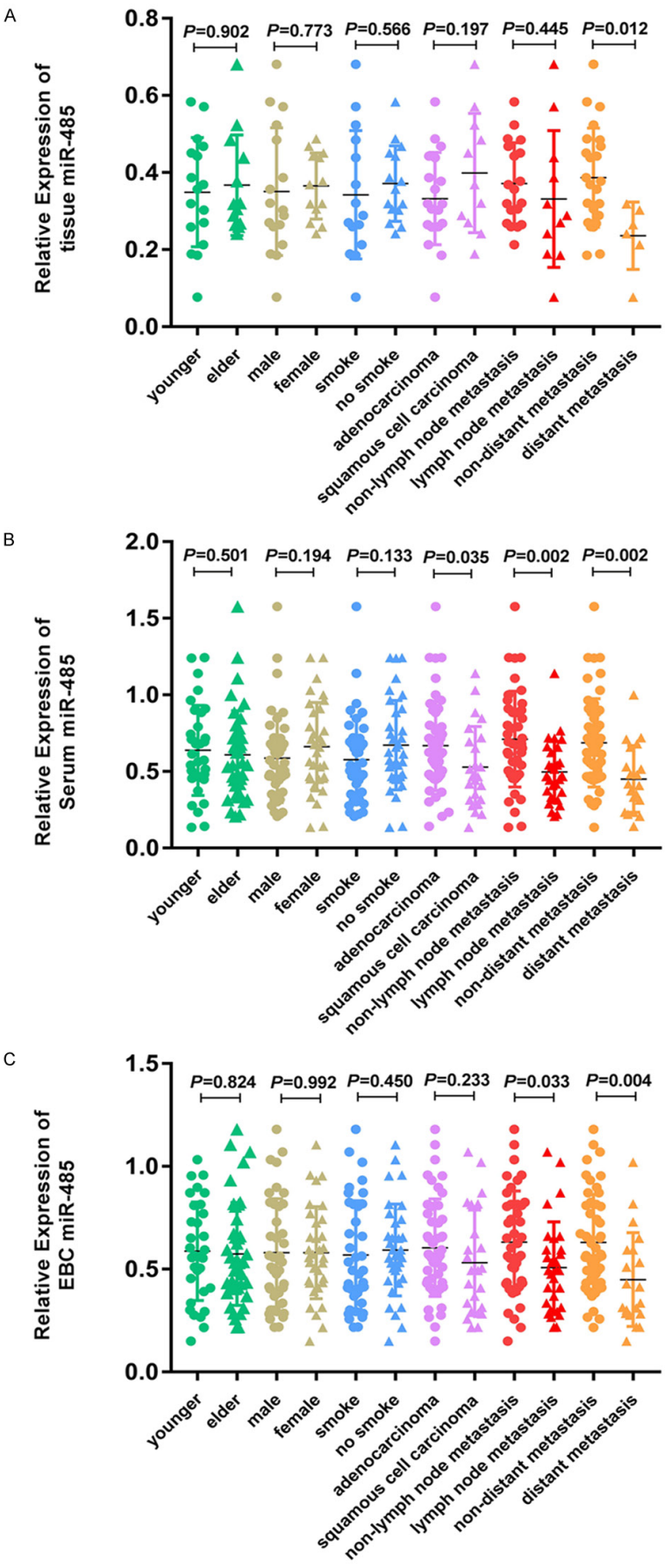

Figure 4. A. Relationship between tissue miR-485 and the clinicopathological characteristics of 30 patients with NSCLC; B. Relationship between serum miR-485 and the clinicopathological characteristics of 72 patients with NSCLC; C. Relationship between EBC miR-485 expression and the clinicopathological characteristics of 72 patients with NSCLC. Elder, > 65 years; younger, ≤ 65 years.

selected as an internal control for tissue samples, and cel-miR-39 was utilized to be an external reference for serum and EBC. The $2^{-\Delta\Delta Ct}$ method was adopted to measure the relative expression level of miR-485.

Statistical analysis

All statistical analysis was performed by IBM SPSS 23.0 and MedCalc, and graphing was performed by GraphPad Prism 7. Whether the data was consistent with the normal distribution was judged by Kolmogorov-Smirnov test or Shapiro-Wilk test. When the data conform to the normal distribution and the homogeneity of the variance is satisfied, the t-test is used to compare the means between the two groups. Measurement data for normal distribution are presented as mean ± standard deviation. Count data were compared by Pearson chi-square test or continuity-corrected chi-square test. Correlation analysis was evaluated by Pearson correlation analysis. Receiver operating characteristics (ROC) curve was applied to assess the diagnostic efficacy of miR-485 in patients with NSCLC. The Z test was used to compare different AUCs in the ROC analysis. If $P < 0.05$ is satisfied, we assume that there is a statistically significant difference.

Table 2. Association of serum miR-485 with clinical laboratory indexes in NSCLC patients

Index	N	serum miR-485	Index	N	serum miR-485
Leukocytes (10^9/L)			CYFRA21-1 (ng/ml)		
0-10	47	0.601±0.246	0-20	64	0.645±0.291
≥ 10	25	0.661±0.361	≥ 20	8	0.438±0.212
P value		0.837	*P* value		0.057
NLR			CEA (ng/ml)		
< 5	51	0.618±0.301	0-5	39	0.684±0.291
≥ 5	21	0.631±0.267	≥ 5	33	0.549±0.275
P value		0.869	*P* value		0.018
PLR			SCC (ng/ml)		
< 200	53	0.662±0.289	0-1.5	48	0.660±0.275
≥ 200	19	0.510±0.268	≥ 1.5	24	0.546±0.310
P value		0.038	*P* value		0.118

Note: NLR, neutrophil-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; CYFRA21-1, cytokeratin 19 fragments; CEA, carcinoembryonic antigen; SCC, squamous cell antigen.

Results

Expression levels of miR-485 among patients with NSCLC

The levels of miR-485 in tissues, serum and EBC of NSCLC patients were determined by qRT-PCR, as well as healthy individuals, to evaluate whether they were abnormally expressed. As we can see from **Figure 1A**, **1B**, the levels of miR-485 in tissues from the NSCLC group were markedly decreased contrasted with adjacent non-cancerous tissues ($P < 0.05$). In addition, the expression level of miR-485 at stage I-II was increased compared with stage III-IV ($P < 0.05$). The relative level of serum miR-485 was obviously decreased in the NSCLC than that in the control group ($P < 0.05$, **Figure 2A**), and the level of miR-485 in stage I-II was greatly elevated than that in stage III-IV ($P < 0.05$, **Figure 2B**). Compared with the control group, the EBC miRNA485 expression in the NSCLC patients was greatly reduced ($P < 0.05$, **Figure 3A**). Lower levels of EBC miR-485 were found in stages III-IV than that in stages I-II ($P < 0.05$, **Figure 3B**).

Association of miR-485 with clinical characteristics in NSCLC patients

The level of miR-485 from tissue in the group without distant metastasis was increased compared with the group with distant metastasis ($P = 0.012$). No significant differences between other clinicopathological features were identified (**Figure 4A**). As shown in **Figure 4B**, the expression of serum miRNA485 was obviously associated with the pathological type ($P = 0.035$), distant metastasis ($P = 0.002$) and lymph node metastasis ($P = 0.002$), but not to age, gender, and smoking history. Regarding the expression level of EBC miR-485, it was greatly associated with distant metastasis ($P = 0.004$), as well as lymph node metastasis ($P = 0.033$). On the contrary, it is not linked to age, gender, smoking history and the pathological type (**Figure 4C**).

Association of miR-485 with clinical laboratory indexes in NSCLC patients

The correlation between serum miRNA485 and several clinical laboratory indexes among NSCLC patients was examined. It is shown that serum miR-485 expression was significantly related with platelet-lymphocyte ratio (PLR) and CEA levels ($P < 0.05$), but not to neutrophil-lymphocyte ratio (NLR), leukocytes, CYFRA21-1, and SCC ($P > 0.05$) (**Table 2**).

Correlation among the expression of miR-485 in EBC, tissues and blood of patients with NSCLC

According to Pearson chi-square test, we obtained a positive correlation between miR-485 expression in serum and in EBC ($r = 0.770$, $P < 0.05$; **Figure 5A**). Similarly, the miR-485 expression in EBC was positively correlated with that in tissues ($r = 0.828$, $P < 0.05$; **Figure 5B**). A

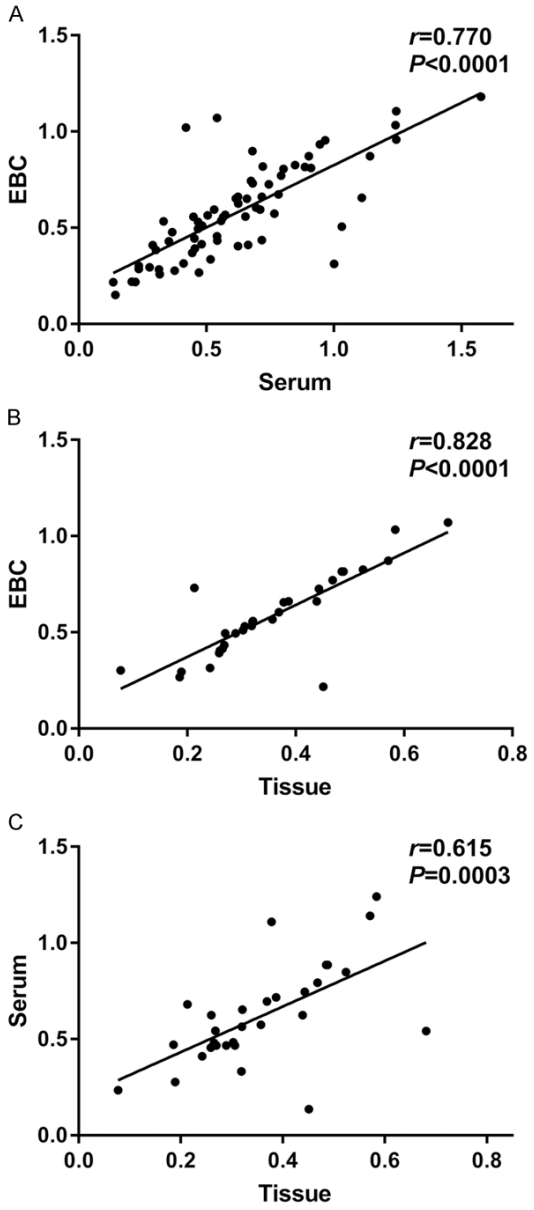

Figure 5. A. Correlation between miR-485 expression level in serum and EBC of 72 patients with NSCLC; B. Correlation between miR-485 expression level in tissues and EBC of 30 patients with NSCLC; C. Correlation between miR-485 expression level in serum and tissues of 30 patients with NSCLC.

positive correlation between miR-485 expression in tissues and in serum was also found (r = 0.615, P < 0.05; **Figure 5C**).

The diagnosis role of miR-485 for NSCLC

We evaluated the diagnostic efficacy of detecting miR-485 in tissues, serum and EBC among the 30 patients by ROC curve analysis. The AUC of serum miR-485 was 0.879 (P < 0.001). At a threshold of 0.791, the specificity and sensitivity were 83.3% and 76.7%, respectively. The AUC of EBC miR-485 was 0.937 (P < 0.001). At a threshold of 0.788, the specificity and sensitivity were 86.7% and 80.0%, respectively. The AUC of tissue miR-485 was 0.943 (P < 0.001). At a threshold of 0.524, the specificity and sensitivity were 90.0% and 90.0%, respectively (**Figure 6**; **Table 3**). The result of the Z test showed that no obvious differences were found in the three AUCs of serum, EBC, and tissue (**Table 4**). Then we evaluated the diagnostic efficacy of detecting miR-485 in serum and EBC among 72 patients. The AUC of serum miR-485 was 0.856 (P < 0.001). At a threshold of 0.721, the specificity and sensitivity were 88.4% and 72.2%, respectively. The AUC of EBC miR-485 was 0.903 (P < 0.001). At a threshold of 0.760, the specificity and sensitivity were 89.9% and 75.0%, respectively. The AUC of the combination was 0.910 (P < 0.001). At a threshold of 0.342, the specificity and sensitivity were 92.8% and 76.4%, respectively (**Figure 7**; **Table 5**). As shown in **Table 6**, the Z test showed that the AUC of miR-485 in EBC was greatly enhanced contrasted with that in the serum (P = 0.029).

Discussion

Lung cancer is a malignant tumor with high morbidity and mortality. The latest statistics show that there were 2.1 million new cases of lung cancer in the world in 2018, accounting for 11.6% of all cancers, and 1.8 million deaths in 2018, accounting for 18.4% of the total cancer deaths [20]. Although new clinical technology for lung cancer has increasingly improved in the past few years, the survival rate of NSCLC patients remains unoptimistic. Lung cancer causes considerable physical and mental pain to patients, and imposes a heavy burden on social and medical resources. During treatment of lung cancer, the survival time of patients should be maximized, and their quality of life should be improved as much as possible. Moreover, EBC is a new biomarker detection method and is prospective for the diagnosis and evaluation of NSCLC.

miRNAs can be partially or entirely complementary to the 3' or 5' untranslated region of their

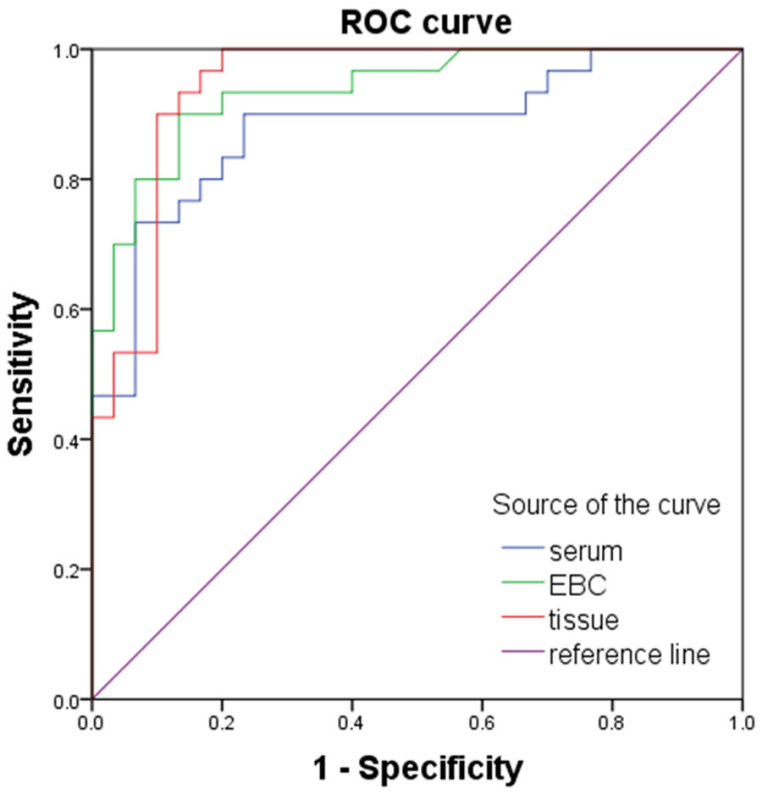

Figure 6. ROC curve of detecting miR-485 in serum, EBC and tissues for diagnosis of NSCLC. Take the value at the maximum Youden index as the cut-off value, and calculate and compare the sensitivity and specificity accordingly.

target gene mRNA through base complementation [21]. The target gene mRNA is spliced, or mRNA translation is inhibited, and thereby the target gene and various important physiological and pathological processes in gene expression regulation can be regulated [22]. Multiple miRNAs are abnormally expressed in tumors [23, 24]. What's more, miRNAs participate in regulating a variety of cellular functions, including apoptosis, metabolism, proliferation, and differentiation. In addition, miRNAs are a participant in the communication of tumor microenvironment cells, they affect the tumor microenvironment, and participate in tumor-related inflammation, hypoxia, and immunity [25, 26].

miR-485 plays an important regulatory role in a variety of tumors. Chai et al. screened miR-485 through miRNAs microarray analysis for further basic research in colorectal cancer cells and found that miR-485 was significantly down-regulated. Besides, miR-485 inhibited proliferation ability of the colorectal cancer cell and was associated with poor prognosis [27]. After that, Han et al. proposed that miR-485 can also inhibit the proliferation and invasion of esophageal cancer cells [28]. In order to investigate whether miRNAs are dysregulated in NSCLC, Peng et al. detected the expression levels of 11 serum miRNAs by qRT-PCR, and the results showed that miR-485 expression levels were significantly down-regulated, which is expected to become a new diagnosis target for lung cancer [29]. Mou et al. proved that miR-485 can inhibit epithelial to mesenchymal (EMT) and metastasis ability of lung adenocarcinoma cells by targeting flotillin-2 (flot-2) [30]. A recent study revealed that miR-485 can inhibit insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP2) from overexpression in NSCLC, thereby inducing the biological effect of tumor cells [31]. Furthermore, miR-485 is also closely related to cisplatin resistance in patients with NSCLC [32]. The above research shows that miR-485 is expected to become a potential new diagnosis biomarker for lung cancer, and it will also help to increase the sensitivity of patients to conventional chemotherapy and improve the poor prognosis.

In the present study, the relative expression of miR-485 was significantly down-regulated in serum, EBC and tissues of patients with NSCLC ($P < 0.05$). miR-485 was significantly associated with TNM stage, lymph node metastasis, and distant metastasis, but not related to age, gender, smoking history, and pathological type. Based on the detection of three types of samples, miR-485 level in patients at stage I-II was significantly lower than that in controls, suggesting that miR-485 expression was significantly reduced in the early stages of NSCLC. With the progress of the disease, the level of miR-485 in patients at stage III-IV was signifi-

Table 3. Diagnostic efficacy of miR-485 in serum, EBC and tissues for NSCLC

group	cutoff value	AUC	*P* value	95% CI		sensitivity	specificity	Youden's index
				lower	upper			
serum	0.791	0.879	< 0.001	0.789	0.969	76.7%	83.3%	0.600
EBC	0.788	0.937	< 0.001	0.880	0.995	80.0%	86.7%	0.667
tissue	0.524	0.943	< 0.001	0.885	1.000	90.0%	90.0%	0.800

Table 4. Comparison of the AUC for detecting miR-485 in serum, EBC and tissues

group	AUC	95% CI	*Z* value	*P* value
serum vs EBC	0.879, 0.937	-0.0121-0.129	1.624	0.104
serum vs tissue	0.879, 0.943	-0.0398-0.169	1.212	0.226
EBC vs tissue	0.937, 0.943	-0.0722-0.0844	0.153	0.878

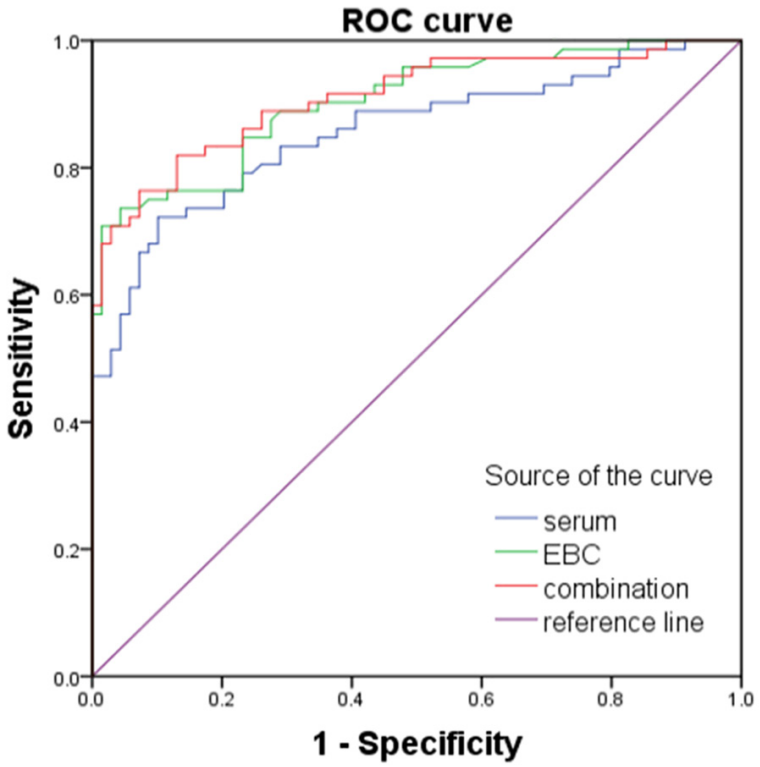

Figure 7. ROC curve of detecting miR-485 in serum, EBC and the two combined for diagnosis of NSCLC. Then the sensitivity and specificity were compared.

cantly lower than that in patients at stage I-II. This may be related to the slow release of miR-485 into the body fluid during the deterioration process, which accumulates into different loadings, so it can be used to preliminary judge the severity of NSCLC patients to a certain extent. We performed correlation analysis on the expression of miR-485 in the EBC, serum and tissues, respectively. The results all showed that they were positively correlated. It suggests that miR-485 is stably expressed in body fluids.

In this study, ROC curve and Z test were used to further evaluate the diagnostic efficacy of detecting miR-485 in serum and EBC of patients with NSCLC. As is shown, compared with that in serum, the detection of miR-485 in EBC has higher AUC, specificity and sensitivity, and the diagnostic value is greater. It suggests that the detection of miR-485 in EBC has obvious advantages over that in serum and may become a potential diagnostic target for lung cancer.

Several recent studies have reported that NLR and PLR affect the survival rate of patients with lung cancer [33, 34], and play a vital role in helping to judge the prognosis of patients with lung cancer. It is demonstrated that serum miR-485 is also closely related to PLR. This may be inseparable from the important role of miRNAs in the tumor microenvironment, suggesting that miR-485 may also affect the overall survival rate.

Tumor markers have been widely used clinically to diagnose lung cancer and predict patient prognosis, such as CEA, CYFRA21-1, and SCC. Previous studies have shown that the above tumor markers are significantly elevated in

Table 5. Diagnostic efficacy of miR-485 in serum, EBC and combination for NSCLC

group	cutoff value	AUC	*P* value	95% CI		sensitivity	specificity	Youden's index
				lower	upper			
serum	0.721	0.856	< 0.001	0.793	0.919	72.2%	88.4%	0.606
EBC	0.760	0.903	< 0.001	0.855	0.952	75.0%	89.9%	0.649
combination	0.342	0.910	< 0.001	0.862	0.958	76.4%	92.8%	0.691

Table 6. Comparison of the AUC for detecting miR-485 in serum, EBC and the combination

group	AUC	95% CI	*Z* value	*P* value
serum vs EBC	0.856, 0.903	0.00476-0.0902	2.178	0.029
serum vs combination	0.856, 0.910	-0.00214-0.111	1.886	0.059
EBC vs combination	0.903, 0.910	-0.0134-0.0269	0.656	0.512

NSCLC [35, 36]. This research discussed the differences in serum miR-485 levels between the three tumor markers and NSCLC patients. The results showed that miR-485 was only related to CEA ($P < 0.05$). Due to the lack of a sufficient number of patients with squamous cell carcinoma, it can affect the correlation between SCC and serum miR-485. The number of squamous cell carcinoma samples needs to be expanded for further analysis.

This work is a retrospective study with insufficient clinical data, that is, the patients with NSCLC receiving chemotherapy or target gene therapy should be followed-up until mortality. In addition, it can also be grouped according to the detailed pathological stage or degree of differentiation to achieve strict and precise grouping of patients with lung cancer.

In summary, our study shows that miR-485 expression is obviously down-regulated in patients with NSCLC and is closely related to clinical stage. It is worth noting that the detection of miR-485 in EBC has a higher diagnostic efficiency. This finding indicates that the detection of miR-485 in EBC is feasible, which is prospective to serve as an early screening biomarker and therapeutic target for NSCLC.

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Disclosure of conflict of interest

None.

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