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Predicative values of serum microRNA-22 and microRNA-126 levels for non-small cell lung cancer development and metastasis: a case-control study

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Presented study aims to explore the predictive values of serum microRNA-22 (miR-22) and miR-126 levels for non-small cell lung cancer (NSCLC) development and metastasis.

A total of 127 NSCLC patients who were admitted in the First People's Hospital of Yancheng City from May, 2013 to May, 2015 were selected as the case group, including 71 cases of adenocarcinoma and 56 cases of squamous cell carcinoma. There were 112 healthy individuals selected as the control group. The qRT-PCR was performed to testify the serum miR-22 and miR-126 levels. Logistic regression analysis was conducted to analyze independent factors influencing NSCLC metastasis and receiver operating characteristic (ROC) curve was drawn to analyze the sensitivity and specificity of serum miR-22 and miR-126 levels in predicting NSCLC developments and metastasis.

The serum miR-22 level was significantly higher in the case group than that in the control group, while the serum miR-126 level was lower in the case group as compared with that in the control group. Compared with squamous cell carcinoma patients, serum miR-22 level significantly increased, while serum miR-126 level decreased in patients with adenocarcinoma. Patients at III + IV stage showed increased serum miR-22 level and relatively decreased serum miR-126 level as compared to patients at I + II stage. Serum miR-22 level elevated in patients with metastasis; in contrast serum miR-126 level reduced in comparison to those without metastasis. In patients with familial inheritance, serum miR-22 level increased but serum miR-126 level decreased as compared to those without familial inheritance. The specificity and sensitivity of serum miR-22 and miR-126 levels in predicting NSCLC development were 99.11%, 84.30%, 82.68% and 96.40%, respectively. The specificity and sensitivity of serum miR-22 and miR-126 levels in predicting NSCLC metastasis were 59.74%, 96.00%, 84.00% and 62.30%, respectively.

Results indicated that serum miR-22 and miR-126 levels may be used as the predicative biomarkers for NSCLC development and metastasis.

Key words: microRNA-22, microRNA-126, non-small cell lung cancer, development, metastasis, predictive value, case-control study

Lung cancer is the major cause of cancer deaths worldwide [1]. Although advances in radio-therapeutics, surgical and chemotherapeutic approaches have been made, the long-term survival rate remains low [2]. Traditionally, decisions on lung cancer therapy have been based on histological considerations, and tumors are assigned to two histological types: non-small cell lung cancer (NSCLC) and small-cell lung cancers [3]. NSCLC comprises three different subtypes: large-cell carcinoma, squamous-cell carcinoma and adenocarcinoma [4]. Tumor extension is one of the major clinical determinants in NSCLC prognosis, which is roughly characterized by the stage [1]. If the cancer is diagnosed at the localized stage, the 5-year survival rate is about 50%, whereas with lymph node involvement or metastasis, the rate decreases precipitously [5]. It has been suggested that discovery and application of molecular biomarkers together with prognosis signature and

traditional cancer staging could improve the management of the NSCLC patients [6]. Progress in genomics and proteomics has generated many new biomarkers with potential clinical values, particularly the gene expression profiling by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) [7, 8]. In recent years, the discovery of microRNAs (miRs) has provided new methods for cancer diagnosis and prediction of treatment response [9, 10].

MiRs are a group of naturally occurring small non-coding RNAs that negatively regulate the translation and stability of target protein-coding mRNAs at the 3' untranslated region (UTR) [11]. A single miR can regulate a number of downstream genes with different biologic entities, therefore the information obtained from miR profiling could provide more accurate classification of cancer subtypes than expression profiles of protein-coding genes [10, 12]. The human miR-22 gene is located at a fragile cancer-related genomic region in chromosome 17 (17p13.3), and mapped to an exon of the C17 or f91 gene [13]. MiR-22 is up-regulated in human epithelial cells and senescent fibroblasts but down-regulated in many cancer cell lines and the over-expression of miR-22 induces acquisition of a senescent phenotype and growth suppression in human normal and cancer cells [11]. MiR-126 is located within intron 7 of EGFL7, an epidermal growth factor-domain gene, which is highly expressed in highly vascularized tissues and endothelial cells, and it controls an essential step in vasculogenesis [14]. Furthermore, miR-126 has been reported to impair tumor progression through signaling pathways related to tumor cell proliferation, invasion, survival and migration [15]. Liu and Lei have found that miR-21 and miR-205 play a role in NSCLC cells' growth, metastasis and sensitivity to radiotherapy and chemotherapy [16, 17]. However, few studies have been addressed about the effects of serum miR-22 and miR-126 levels on NSCLC development and metastasis. In the present study, predictive values of serum miR-22 and miR-126 levels in NSCLC development and metastasis are discussed in order to provide a novel perspective for the diagnosis and treatment for the NSCLC patients.

Materials and methods

Ethics statement. The study was approved by the Ethic Committee and was conducted in accordance with the Ethical Principles. The process of blood samples collection completely complied with relevant rules released by the Ethic Committee of the First People's Hospital of Yancheng City, and both the NSCLC patients and healthy individuals signed informed consent at will before the sample collection.

Participants. Altogether 127 NSCLC patients treated in the First People's Hospital of Yancheng City from May, 2013 to May, 2015 were collected in this study as the case group, and all of them were preliminary diagnosed with primary NSCLC according to the cytopathology and histopathology. Among the patients, there were 82 males and 45 females with the mean age of 54.9 ± 10.8 years old; according to pathological types, there were 71 cases of adenocarcinoma and 56 cases of squamous cell carcinoma; according to tumor node metastasis (TNM) staging standard released by Union for International Cancer Control (UICC, the 7th version in 2009) [18], 29 of them were at I stage (22.83%), 21 at II stage (16.54%), 64 at III stage (50.39%) and 13 at IV stage (10.24%); there were 50 patients with metastasis and 77 patients without metastasis. A total of 112 healthy individuals were selected as the control group, including 63 males and 49 females, with the mean age of 44.3 ± 10.4 years old. No significant difference of gender and age was found between the control and case groups. The inclusion criteria for NSCLC patients: patients who had not received chemotherapy or radiotherapy; patients without tumor-related disease history; patients diagnosed with NSCLC by postoperative pathological examination. The inclusion criterion for healthy individuals: no tumor-related diseases was found by chest computed tomography (CT), chest X ray, blood test and other general physical tests.

Collection of blood samples and extraction of circulating microRNA. On the second day of admission, whole blood was drawn from all patients. And the drawn blood samples were treated with ethylene diamine tetra-acetic acid (EDTA)-K2 anticoagulant and centrifuged to separate plasma, which was dispensed into tubes with 500 μl per tube and reserved at -80°C for the later use. A volume of 500μl plasma sample reserved at low temperature was collected and fully mixed with the trizolls (Invitrogen Inc., Carlsbad, CA, USA) which was 3 times the volume of plasma and was melted on ice. Afterwards, the plasma was maintained at room temperature for 15 min and mixed with 0.5 ml chloroform. Then, the plasma was maintained at room temperature again for 5 min. At 4 °C, the plasma was centrifuged at 14000 r/min for 15 min and the upper liquid was transferred into a new tube, after which anhydrous ethanol (1.5 times the volume of the liquid) was poured into the tube. According to the specification of miRNeasy mini kit (TIANGEN Biotechnology Co. Ltd, Beijing, China), RNA was extracted with an adsorbent column. The extracted RNA was dissolved in 500 μl h2o (RNAase-free). Concentration and purity of the RNA were detected by ultraviolet spectrophotometry and integrity of the RNA was observed by 3% agarose gel electrophoresis.

Quantitative real-time fluorescent polymerase chain reaction (qRT-PCR). MiR molecular was treated with 3' end poly (A) tract and with reverse transcription according to the specifications of miR reverse transcription cDNA synthesis kit (KR201) (TIANGEN Biotechnology Co. Ltd, Beijing, China). With the synthesized cDNA as the template, qRT-PCR was conducted with taqman® PCR reaction kit provided by Thermo Fisher Scientific (California, USA); besides, miR-16 was regarded as an internal reference (primer sequence was shown in Table 1). Reaction condition was as follows: pre-denatured at 95°C for 1 min, denatured at 95°C for 15 s, annealed at 60°C for 45 s, extended at 72°C for 40 s and 40 cycles in total. In qRT-PCR, repeated and negative controls experiments were conducted, in which each sample was repeated for 3 times in

quantitative experiment and no cDNA template was added in negative control experiment. The relative expressions of miR-22 and miR-126 were shown by $2^{-\Delta\Delta Ct}$. In each sample, the $\Delta Ct = Ct_{\text{targeted gene}} - Ct_{\text{internal reference gene}}$ and $\Delta\Delta CT = \Delta Ct_{\text{experiment group}} - \Delta C\,t_{\text{control group.}}$ Ct value represented fluorescence intensity value detected by thermocycler in the reaction. In order to check the integrity of PCR amplification, 3% agarose gel electrophoresis was used to analyze and verify the specificity of PCR product.

Statistical analysis. SPSS 21.0 software (SPSS Inc.; Chicago, IL, USA) was employed for statistical analysis, in which measurement data was shown by $\bar{x} \pm s$ and comparison between two groups was tested by two tail t-test. Logistic regression analysis was adopted to analyze independent factors influencing NSCLC metastasis and ROC curve was used to analyze the sensitivity and specificity of serum miR-22 and miR-126 levels in predicting NSCLC developments and metastasis. $P < 0.05$ indicated significant difference.

Results

Comparison of serum miR-22 and miR-126 levels between the case and control groups. The serum miR-22 level in the case group was significantly higher than that in the control group ($P < 0.05$) (Figure 1); while the serum miR-126 level in the case group was obviously lower than that in the control group ($P < 0.05$).

Table 1. PCR primer sequences of microRNA-22, microRNA-126 and microRNA-16.

Primer	Sequence
MicroRNA-22	F:5`-GCCTGAAGCTGCCAGTTGA-3`
	R:5`-GTGCAGGGTCCGAGGT-3`
MicroRNA-126	F:5`-CGCTGGCGACGGGACATTATTAC-3`
	R:5`-TGCCGTGGACGGCGCATTATTAC-3`
MicroRNA-16	F:5`-GCGGATCCAGCACATCATGGTTTACA-3`
	R:5`-GCGTCGACAAAAAATGTTACCTTAAAGGG-3`

PCR, polymerase chain reaction; F, forward; R, reverse.

Comparisons of serum miR-22 and miR-126 levels in the NSCLC patients with different clinical features. There were significant differences of serum miR-22 and miR-126 levels in terms of pathological types, TNM staging, with or without metastasis and with or without familial inheritance (both $P < 0.05$). Compared with squamous cell carcinoma patients, serum miR-22 level significantly increased, while serum miR-

Table 2. Comparisons of serum microRNA-22 and microRNA-126 levels in the NSCLC patients with different clinical features.

Feature	N	MicroRNA-22			MicroRNA -126		
		Level	t	*P*	Level	t	*P*
Age (year)							
< 60	85	1.35 ± 0.33	0.000	0.999	0.63 ± 0.26	0.403	0.688
≥ 60	42	1.35 ± 0.41			0.61 ± 0.27		
Gender							
Male	82	1.35 ± 0.37	0.153	0.879	0.64 ± 0.24	0.625	0.533
Female	45	1.34 ± 0.32			0.61 ± 0.29		
Pathologic types							
Adenocarcinoma	71	1.40 ± 0.40	2.085	0.039	0.57 ± 0.23	2.652	0.009
Squamous cell carcinoma	56	1.27 ± 0.27			0.69 ± 0.28		
TNM staging							
Stage I + II	50	1.10 ± 0.30	7.679	< 0.001	0.75 ± 0.27	4.572	< 0.001
Stage III + IV	77	1.51 ± 0.29			0.55 ± 0.22		
Metastasis							
Yes	50	1.52 ± 0.23	4.847	< 0.001	0.49 ± 0.18	5.073	< 0.001
No	77	1.23 ± 0.38			0.71 ± 0.27		
Obstructive pneumonia							
Yes	62	1.38 ± 0.34	0.950	0.344	0.61 ± 0.28	0.866	0.388
No	65	1.32 ± 0.37			0.65 ± 0.24		
Familial inheritance							
Yes	15	1.75 ± 0.29	4.031	< 0.001	0.47 ± 0.22	3.194	0.002
No	112	1.29 ± 0.33			0.66 ± 0.26		
Smoking history							
Yes	91	1.34 ± 0.32	0.284	0.777	0.63 ± 0.26	0.260	0.819
No	36	1.36 ± 0.44			0.63±0.26		

NSCLC, non-small cell lung cancer; TNM, tumor node metastasis; N, number; t: Student's *t*-test; *P*, probability; *t* values and *P* values were compared between groups.

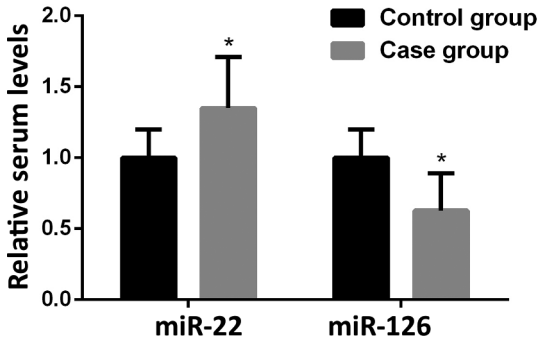

Figure 1. Comparison of serum microRNA-126 and microRNA-22 levels between the case and control groups (` x ± s). NSCLC, non-small cell lung cancer; N, number; *compared with the control group, $P < 0.05$.

126 level decreased in patients with adenocarcinoma. Patients at III + IV stage showed increased serum miR-22 level and relatively decreased serum miR-126 level as compared to patients at I + II stage. Serum miR-22 level elevated in patients with metastasis; in contrast serum miR-126 level reduced in comparison to those without metastasis. In patients with familial inheritance, serum miR-22 level increased but serum miR-126 level decreased as compared to those without familial inheritance. However, no significant differences were found in age, gender, obstructive pneumonia and smoking history (all $P > 0.05$) (Table 2).

Logistic regression analysis for independent factors influencing NSCLC metastasis. Single-factor logistic regression analysis of general information of 50 patients with metastasis and 77 patients without metastasis was carried out, including patient's age, gender, smoking history, family inheritance, with or without obstructive pneumonia, pathological types, TNM staging, and serum miR-22 and miR-126 levels. It was indicated that comparisons in term of family inheritance, pathological types, TNM staging and serum miR-22 and miR-126 levels showed significant differences. Postoperative recurrence and metastasis as dependent variable and significant factors of the single-factor analysis as independent variable were included in the multi-factor logistic regression analysis, which illustrated that TNM staging and pathologic types of NSCLC, serum miR-22 and miR-126 levels could be prognostic factors of the NSCLC patients with metastasis (all $P < 0.05$) (Table 3).

ROC curve analysis for serum miR-22 and miR-126 levels in predicating NSCLC development and metastasis. ROC curve was drawn from the comparison of 127 NSCLC patients and 112 healthy participants. The area under the curve (AUC) of serum miR-22 and miR-126 concentrations of were 0.916 (95%CI: 0.872-0.960, $P < 0.001$) and 0.874 (95%CI: 0.820-0.927, $P < 0.001$). The specificity and sensitivity of serum miR-22 and miR-126 levels in NSCLC diagnosis were 99.11% and 84.30%, 82.68% and 96.40%, respectively at the optimal cutoff value, when relative expression levels of serum miR-22 and miR-126 were 1.07 and 0.95 (Figure 2A, B). ROC curve was drawn based on the comparison of 50 patients with metastasis and 77 patients without metastasis of NSCLC patients. The AUC of miR-22 and miR-126 concentrations were 0.788 (95%CI: 0.710-0.865) and 0.746 (95%CI: 0.661-0.831). The specificity and sensitivity of serum miR-22 and miR-126 in NSCLC diagnosis were 59.74% and 96.00%, 84.00% and 62.30% respectively at the optimal cutoff value, when relative levels of serum miR-22 and miR-126 were 1.30 and 0.63 (Figure 2C, D).

Discussion

Approximately 65% of NSCLC patients have advanced disease at diagnosis and most NSCLC cases rarely show symptoms and are difficult to be detected, particularly at Stages I and II [19]. Early detection represents a promising strategy to reduce NSCLC mortality [20]. The reference standard in diagnosing NSCLC is pathologic evidence of malignant cells, which typically requires invasive strategies, such as transthoracic needle aspiration, bronchoscopy or thoracotomy [19]. Therefore, it is important to develop new methods and biomarkers with specificity and sensitivity for the detection of NSCLC. In the present study, the serum miR-22 and -126 levels in predicating NSCLC developments and metastasis were analyzed in order to provide a new method for NSCLC diagnosis and treatment.

In the study, the elevated serum miR-22 level was observed in the NSCLC patients. MiR-22, a 22-nt non-coding RNA original-

Table 3. Logistic regression analysis of the independent factors influencing NSCLC metastasis

Factor	B	S.E.	Wald	Sig.	Exp(B)	95%CI for Exp(B)	
						Lower	Upper
Familial inheritance	- 2.291	0.847	7.313	0.007	0.101	0.019	0.532
Pathologic types	- 2.491	0.735	11.488	< 0.001	0.083	0.020	0.350
TNM staging	- 1.913	0.865	4.889	0.027	0.148	0.027	0.805
MicroRNA-22 level	2.930	1.129	6.733	0.009	18.723	2.048	171.161
MicroRNA-126 level	- 3.596	1.196	9.046	0.003	0.027	0.003	0.286

NSCLC, non-small cell lung cancer; TNM, tumor node metastasis; B, partial regression coefficient; S.E., standard error; Sig., significance; Exp(B), exponent function (partial regression coefficient); 95%CI, 95% confidence interval.

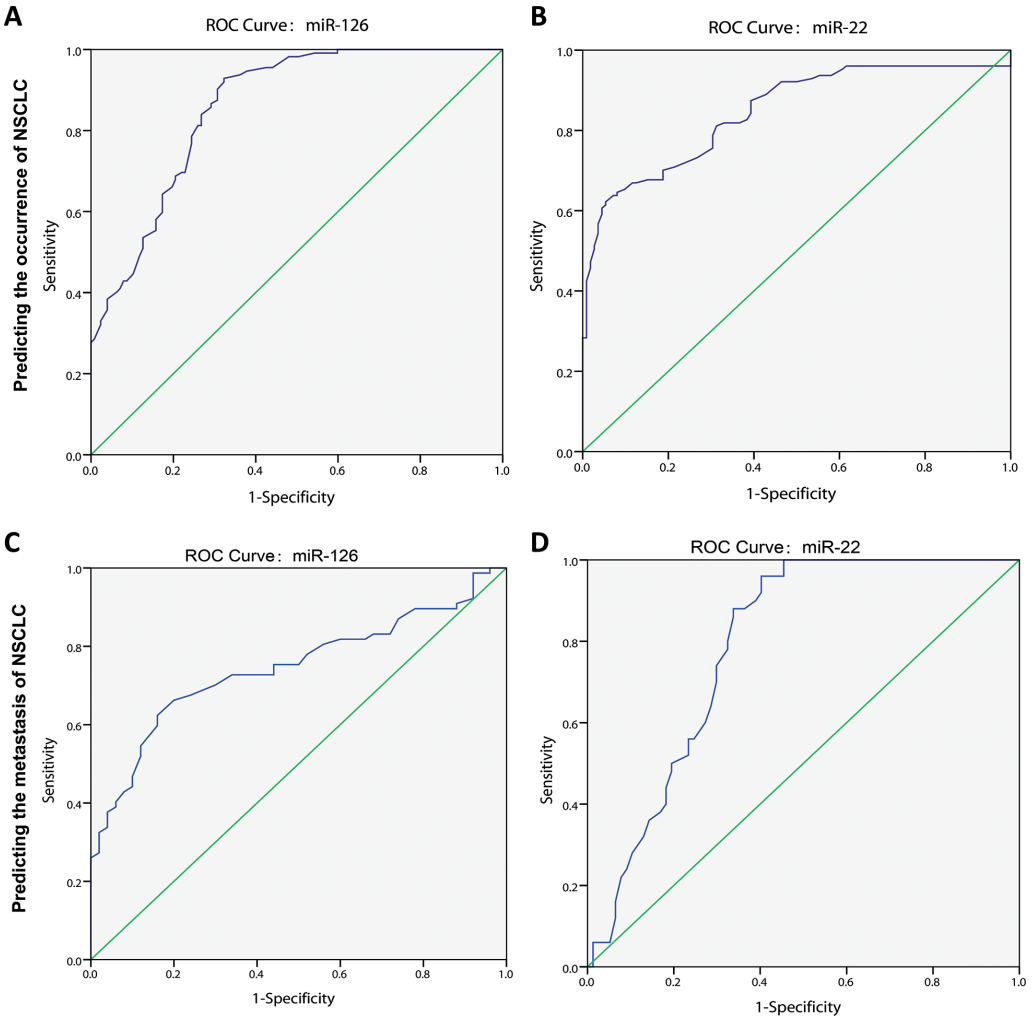

Figure 2. ROC curve analysis for serum microRNA-22 and microRNA-126 levels in predicating NSCLC development and metastasis.
A, specificity and sensitivity of serum miR-126 level in predicating NSCLC development; B, specificity and sensitivity of serum miR-22 level in predicating NSCLC development; C, specificity and sensitivity of serum miR-126 level in predicating NSCLC metastasis; D, specificity and sensitivity of serum miR-22 level in predicating NSCLC metastasis.

ly identified in HeLa cells, has been found to be down-regulated in breast cancer, multiple myeloma, cholangiocarcinoma and hepatocellular carcinoma, but over-expressed in prostate cancer [21]. A previous study has suggested that miR-22 functioned in multiple cellular processes, including proliferation, senescence, differentiation and apoptosis, and the deregulation of miR-22 is a hallmark of human cancer [22]. Epithelial to mesenchymal transition (EMT) is a phenotypic manifestation of complex changes in gene expression that include increased expression of mesenchymal markers and decreased expression of epithelial markers [23]. EMT is a key developmental process that cancer cells hijack to increase their invasive potential and aggressiveness [24], and it is mainly characterized by cell-cell adhesion loss, apical-basal cell polarity and the increased motility of cells [25]. In Song's study, it is shown that miR-22 triggers enhanced mammary gland hyperplasia and a marked expansion of the mammary stem cell compartment, hence triggering tumor initiation, and miR-22 promotes the metastatic process and EMT through its ability to repress the expression of 5hmC as well as miR-200s by directly targeting members of the ten eleven translocation (TET) family [26]. A previous study has shown that NSCLC drug resistance and metastasis has been associated with EMT [27]. Therefore, it is believed that the over-expression of miR-22 has been found in NSCLC which is confirmed by Ling Bo's study in which over-expression of miR-22 was demonstrated in patients with NSCLC [21].

In addition, the study showed NSCLC patients had decreased serum miR-126 level compared with healthy individuals. MiR-126 is an endothelial cell-specific miRNA [28]. Previous reports suggested that miR-126 acts as a tumor suppressor by targeting epidermal growth factor like domain 7 (EGFL7), vascular endothelial growth factor A (VEGF-A)

or *SLC7A5* gene which yields growth advantages [15, 29, 30]. VEGF blockade has a rapid and direct anti-vascular effect in human tumors [31]. EGFL7 expression could promotes tumor progression by down-regulate the expression of endothelial molecules that mediate immune cell infiltration [32]; and *SLC7A5* mediates absorption of amino acids, which are used in tumors as oxidative fuel for Adenosine Triphosphate (ATP) production [33]. In addition, Crk, a predicted putative target gene for miR-126, is a member of a family of adaptor proteins that are involved in intracellular signal pathways altering cell adhesion migration and proliferation, and decreased miR-126 in lung cancer cell line results in increased Crk, which is associated with tumor invasion [34]. Consistently, down-regulation of miR-126 in cancerous and noncancerous tissues has been reported for cancers of the lung, cervix, stomach and prostate [14, 35-38], by which the result is confirmed.

Finally the study suggested that serum miR-22 and -126 levels were significantly different in these clinic features including pathological types, TNM staging, metastasis and family inheritance. And the logistic regression analysis further confirmed that TNM staging, pathological types, and serum miR-22 and miR-126 level may be prognostic factors correlated with foci metastasis of NSCLC patients. Meanwhile, existed study has demonstrated that miR-22 in lung cancer patients could be a useful biomarker in the follow-up of early stage NSCLC and for early diagnosis in healthy heavy smokers [39], and that miR-126 is an independent and strong negative prognostic factor in NSCLC, and its prognostic impact appears related primarily to nodal status and histology [40]. Therefore, serum miR-22 and-126 level may serve as molecular diagnostic and prognostic markers for patients with NSCLC.

In conclusion, the study suggested that biomarker miR-22 and -126 were of sensitivity and specificity in predicting NSCLC development and metastasis, thus providing a new method for clinical NSCLC treatment. However, participants collected were patients who were admitted in the First People's Hospital of Yancheng City from May 2013 to May 2015. Thus, the time for follow-up was relatively short and it was unlikely for us to conduct survival analysis. Luckily, we are planning to further our study and add the follow-up in our future research. Nevertheless, the mechanism of correlation between miR-22 and -126 and clinical features of the NSCLC patients remains unknown, and further studies are needed.

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