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Clinical significance of serum miR-25 in non-small-cell lung cancer

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ABSTRACT

Background: MicroRNAs (miRNAs) are becoming recognized as novel diagnostic and prognostic biomarkers in several malignancies, including non-small-cell lung cancer (NSCLC). miR-25 is overexpressed in small cell lung cancer (SCLC) and NSCLC tissues, and high miR-25 expression is associated with poorer overall survival of women with lung ADC. We hypothesised links between serum miR-25 levels and clinicopathological characteristics, diagnosis and prognosis of NSCLC patients.

Methods: Serum miR-25 was determined by real-time quantitative polymerase chain reaction in 128 NSCLC patients and 128 healthy controls, and links between miR-25 level and cliniopathological characteristics including diagnosis and prognosis were explored.

Results: Median (IQR) serum miR-25 levels were significantly increased in NSCLC compared to healthy controls at 0.86 relative units (0.14–1.78) versus 0.23 (0.08–0.96) ($P < 0.001$). Using a cut-off of 0.67 units, miR-25 had a sensitivity of 76.4%, specificity of 84.6%, accuracy of 72.6%, positive predictive value of 92.8% and negative predictive value of 68.5% for the diagnosis of NSCLC. High serum miR-25 level was significantly associated with gender ($P = 0.042$), tumour stage ($P = 0.014$) and lymph node metastasis ($P < 0.001$). In multivariate analyses, miR-25 was an independent prognostic factor for overall survival and relapse-free survival.

Conclusions: Serum levels of miR-25 could improve NSCLC screening, and be a useful diagnostic and prognostic marker of NSCLC.

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Introduction

Lung cancer is a leading cause of cancer-related deaths worldwide, with non-small-cell lung cancer (NSCLC) representing approximately 80% of these lung cases [1]. Most patients are diagnosed at an advanced stage of disease and have little prospect of effective and curative treatment: five-year survival rates are <15% [2]. A promising breakthrough with the potential to improve outcomes for NSCLC patients involves the introduction of validated biomarkers into clinical management. These biomarkers may be crucial not only for early diagnosis, but also to inform treatment decisions to achieve optimal therapeutic interventions [3].

Small non-coding RNAs including microRNAs (miRNAs) have been recently recognized as important regulators of gene expression, with many roles in physiological processes as well as in the pathogenesis of a number of diseases by translational repression or mRNA destabilization of numerous target genes [4]. For example, miR-935 is reduced in NSCLC tissues, and predicts outcome survival [5]. The miR-106b-25 cluster is highly conserved in vertebrates and consists of three members including miR-106b, miR-93 and miR-25. miR-106b and miR-93 share the same seed sequences; however, miR-25 has only a similar seed sequence resulting in different predicted target mRNAs [6].

miR-25 is expressed in a wide variety of tissues and cell types targeting many mRNAs. Both overexpression and repression of miR-25 could result in the development of different diseases [7–9]. miR-25 is linked with carcinogenesis in breast cancer [10], cholangiocarcinoma [11] and ovarian cancer [12]. Other studies suggest that overexpression of miR-25 reduces apoptosis by different mechanisms in human NSCLC cells and cell lines [13,14]. He et al. reported that radiotherapy-resistant NSCLC human tissues overexpressed miR-25 as compared to radiosensitive or non-cancerous tissues [15]. An ethnically diverse multicentre case-control study recruiting 221 NSCLC patients, 161 controls and 56 patients with benign nodules reported that serum levels of miR-25 and other four miRNAs (miR-483-5p, miR-193a-3p, miR-214 and miR-7) were significantly elevated irrespective of ethnicity groups [16]. A clinical study enrolling 100 Chinese female non-smoking lung adenocarcinoma patients found that increased plasma miR-25 levels positively linked with the mortality rate, advanced disease stage, regional and distant metastasis at diagnosis [17]. The high expression of miR-25 was associated with lymph node metastasis and poor long-term survival in patients with GC undergoing radical resection and adjuvant systemic

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chemotherapy [18]. In addition, the combination of the three miRNAs (miR-125a-5p, miR-25 and miR-126) gives an 0.94 area under the receiver operating characteristic curve value in distinguishing early stage lung cancer patients from control subjects with 87.5% sensitivity and 87.5% specificity, respectively [19]. However, the role of serum miR-25 and its clinical significance in patients with NSCLC remains unknown.

We therefore hypothesised (a) a clinical significance of serum miR-25 in patients with NSCLC, and (b) that miR-25 levels have value as a biomarker for diagnosis and prognosis.

Materials and methods

All experiments were performed in accordance with relative regulations and manners. The study was approved by the Clinical Research Ethics Committee of the central hospital of Linyi, Yishui, Shandong, China, from where all clinical samples were collected. All participants in the study gave their written informed consent. The hypotheses were tested in 128 cases diagnosed with NSCLC from April 2009 to January 2011, and 128 healthy controls recruited from individuals visiting the hospital. Clinical and demographic features of cases and controls are listed in Table 1. Cancer stage was determined according to the American Joint Committee on Cancer Guidelines (6th and 7th edition) and the best available (clinical or pathological stage) was used. Most patients with I to IIIA stages underwent surgery for their primary tumours, while most patients with stages IIIB and IV had chemotherapy. Radiotherapy was added if necessary. Patients were followed up for up to 70 months for outcome survival (OS, i.e. death) and relapse-free survival (RFS).

Table 1. Clinical and demographic characteristics of study participants.

	Groups		
Clinical parameters	NSCLCs (n = 128)	Controls (n = 128)	P value
Sex: Male/female	98/30	89.39	0.205
Age (median (range))	63 (31–82)	63 (27–78)	0.637
Smoking status			0.003
Never	51	75	
Previous/current	77	53	
Histological type			
Adenocarcinoma	102	–	
Squamous carcinoma	26	–	
Tumour stage			
I	56	–	
II (IIA/IIB)	38 (22/16)	–	
III(IIIA/IIIB)	29 (13/16)	–	
IV	5	–	
Lymph node metastasis			
Yes	92	–	
No	36	–	
Tumour size (cm)			
≤2	45	–	
>2	83	–	
Differentiation			
Well+ moderate	76	–	
Poor	52	–	

Blood samples were collected after diagnosis: 8.5 ml of peripheral blood was collected in a serum separation tube, was allowed to clot for 30 min at room temperature and then placed on ice. Within 2 h, samples were centrifuged at 12,000 g for 10 min and serum aliquoted and stored at −80 °C until analysis. Total RNA was extracted from 800 µL of serum samples using miRNeasy RNA isolation Kits (Qiagen) according to the manufacturer's protocol.

Six hundred microlitres of serum was used for RNA isolation using a mirVana PARIS RNA isolation kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. The RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Labtech International, Heathfield, UK) and a 15% denatured polyacrylamide gel. The RNA samples were subjected to a reverse transcription reaction using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. qPCR was carried out on the serum samples in triplicate using TaqMan 2× Universal PCR Master Mix with no AmpErase UNG (Applied Biosystems) on an ABI 7500 Real-Time PCR system (Applied Biosystems). qPCR amplification conditions were set to an initial cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The cycle threshold (Ct) values were calculated using SDS 2.0.1 software (Applied Biosystems). No template controls were used in either the RT or PCR steps to ensure target-specific amplification. The control miRNA was U6 small nuclear RNA (snRNA). Levels of serum miR-25 were calculated using the $2^{-\Delta Ct}$ method relative to the average of U6 snRNA [20]. If the mean C_t value for U6 was not between 20 and 32 cycles, the assay was repeated at least once on some of the samples. Samples with low U6 snRNA levels were not included for data analysis in this study.

The statistical analyses were performed using the SPSS software package, version 22.0 (SPSS Inc., Chicago, IL, USA) and MatLab R2012b (The MathWorks Inc., Natick, MA, USA). Unpaired or paired Student t test, Fisher exact test, χ^2test, Mann–Whitney and Kruskal–Wallis test were applied where appropriate to analyse the association between miR-25 and clinicopathologic parameters. The median value of miR-25 as the cut-off value for all analyses. Overall survival was defined as the time from date of diagnosis to the date of death by any cause, and it was assessed using the Kaplan–Meier method. The log-rank test was performed to compare the survival curves of individual groups. Univariate and multivariate analyses were performed for prognostic factors of overall survival using the Cox regression model. The reported results included hazard ratio (HR) and 95% confidence intervals (CI). Receiver operating characteristic (ROC) curves were generated to assess the

diagnostic accuracy of miR-25. Areas under the curves (AUC) were calculated, assuming nonparametric distribution. Two-sided $P < 0.05$ was considered statistically significant.

Results

Cases and controls were matched for age, sex and smoking history (Table 1). Median (IQR) serum miR-25 levels were significantly increased in NSCLC compared to healthy controls at 0.86 relative units (0.14–1.78) versus 0.23 (0.08–0.96) ($P < 0.001$). Links between miR-25 and clinical histopathological features were determined by Mann-Whitney U test (Table 2). There were significant differences between the miR-25 levels and gender, tumour stage and lymph node metastasis, but none between miR-25 and age, smoking history, histology, tumour size and differentiation.

The 128 NSCLC patients were separated into miR-25 high risk (higher levels) and low risk (lower levels) groups using Cutoff Finder software (http://molpath.charite.de/cutoff), to generate the optimum cut-off score for the normalized serum expression ($-\Delta^{Cq}$) [20]. The Kaplan–Meier analysis showed that patients with higher levels of miR-25 have unfavourable overall survival (OS; HR = 2.67, 95% CI = 1.06–6.57, $P = 0.046$) and relapse-free survival (RFS) time (HR = 1.54, 95% CI = 1.18–7.94, $P = 0.036$) in comparison with those patients with low levels of miR-25 (Figure 1(a,b)). Using a cut-off value of 0.67, AUC (95% CI) was 0.62 (0.53–0.69) ($P<0.001$), with sensitivity of 76.4%, specificity of 84.6%, accuracy of 72.6%, positive predictive value of 92.8% and negative predictive value of 68.5% for the diagnosis of NSCLC (Figure 1(c)).

Univariate Cox regression analyses of the prognostic significance showed that male sex, lymph node metastases, tumour size, differentiation, levels of miR-25 and high stage were all predictors of poor survival (Table 3). However, in multivariate analysis, only age, lymph node metastases, tumour size, levels of miR-25 and high stage were independent prognostic factors.

Table 2. Relationship between serum miR-25 and clinicopathological factors in patients with NSCLC.

	Relative expression of serum miR-25	*P*
Sex		0.042
Male ($n = 98$)	0.92 (0.16–1.94)	
Female ($n = 30$)	0.76 (0.11–1.63)	
Age (years)		0.436
≤60 years	0.86 (0.15–1.75)	
> 60 years	0.87 (0.13–1.82)	
Smoking history, *n* (%)		0.108
Never ($n = 27$)	0.86 (0.13–1.79)	
Previous/current ($n = 85$)	0.85 (0.16–1.63)	
Unknown ($n = 16$)	0.87 (0.12–1.74)	
Stage		0.014
I ($n = 56$)	0.76 (0.15–1.47)	
II ($n = 38$)	0.85 (0.17–1.54)	
III ($n = 29$)	0.94 (0.19–1.63)	
IV ($n = 5$)	1.12 (0.27–1.85)	
Histology		0.334
Adenocarcinoma (AC) ($n = 102$)	0.87 (0.13–1.79)	
Squamous carcinoma (SCC) ($n = 26$)	0.85 (0.18–1.72)	
Lymph node metastasis		<0.001
Yes ($n = 92$)	1.13 (0.39–2.21)	
No ($n = 36$)	0.74 (0.12–1.76)	
Tumour size (cm)		0.097
≤2 ($n = 45$)	0.86 (0.13–1.79)	
>2 ($n = 83$)	0.88 (0.16–1.72)	
Differentiation		0.375
Well+moderate ($n = 76$)	0.87 (0.13–1.82)	
Poor ($n = 52$)	0.85 (0.14–1.74)	

Discussion

Serological biomarkers are becoming important in the management of lung cancer [21,22]. Previous clinical studies in NSCLC exposed the pros and cons of common serum biomarkers for diagnostic applications [23–26], while others underlined their value in disease prognosis and therapy monitoring [27–30]. From a clinical standpoint, CYFRA 21–1, CEA, CA-125 and SCC still represent the most valuable markers in NSCLC and are primarily used for disease monitoring. However, their prognostic value remains in doubt. Recently, focus has shifted on the discovery of novel prognostic and monitoring markers, as a means to improve clinical management of NSCLC

Small non-coding RNAs including microRNAs (miRNAs) have been recently recognized as important regulators of gene expression. MicroRNAs play myriads of roles in physiological processes as well as in the pathogenesis of a number of diseases by translational repression or mRNA destabilization of numerous target genes [31]. Furthermore, miRNAs have been shown to be present in a remarkably stable form in plasma/serum, and the expression level of serum/plasma miRNAs is reproducible and consistent among individuals [32]. In fact, a number of reports have suggested the presence of circulating miRNAs and their potential use as novel biomarkers for cancers [6]. MiR-25 is a well-described oncogenic miRNA playing a crucial role in the development of many tumour types [6,33]. Recent reports have shown that circulating miR-25 has diagnostic and/or prognostic value in patients with colorectal cancer [34] and hepatocellular cancer [35].

We confirmed our hypotheses, finding that serum miR-25 is significantly higher in NSCLC patients than that in the healthy controls, speculating that miR-25 acts as a potential tumour-promotor in NSCLC. In addition, the noticeable increased serum miR-25 was found in patients with high-grade tumour and lymph node metastasis, playing a key role in the development and progression of NSCLC. To assess the prognostic value of serum miR-25 in NSCLC, Kaplan-Meier survival curves were constructed and then compared

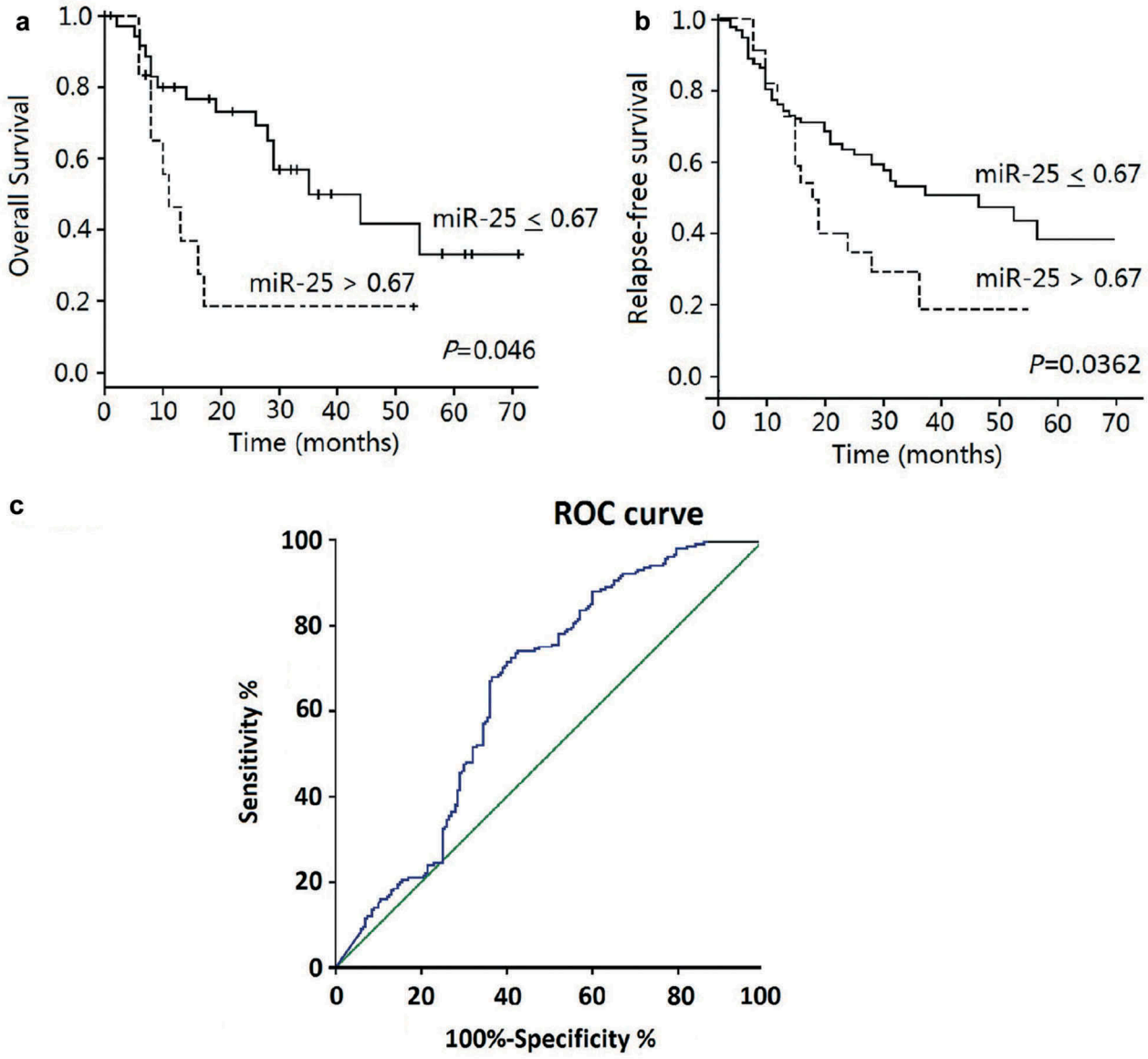

Figure 1. Relationship between miR-25 and survival of patients with NSCLC.

Table 3. Univariate and multivariate analysis of factors associated with survival of patients with non-small-cell lung cancer for overall survival.

	Univariate analysis			Multivariate analysis		
Variable	HR	95%CI	*P*	HR	95%CI	*P*
Age (Y) ≥ 60 vs. < 60	0.49	0.08–1.47	0.356	–	–	–
Sex Male vs. female	2.48	1.48–4.86	0.001	1.68	1.23–2.57	0.01
Smoking history (non vs. current or former smoker)	1.25	0.88–1.71	0.23	–	–	–
Histology SCC vs. AC	1.23	0.86–1.95	0.083	–	–	–
Lymph node metastasis Yes vs. No	6.79	3.18–15.7	<0.001	1.27	1.04–2.39	0.012
Tumour size (cm) ≤2 vs. >2	1.258	1.04–3.29	0.008	1.54	1.14–3.75	0.034
Differentiation Well vs. Moderate vs. poorly	1.387	1.16–3.42	0.025	1.11	0.84–1.45	0.136
Levels of miR-25 ≤ 0.67 vs. > 0.67	4.77	1.74–12.68	0.002	1.53	1.28–3.15	0.037
Stage I+ II vs. III+IV	5.227	2.86–13.43	0.001	5.85	2.93–14.47	0.002

by the log-rank test, and we found that NSCLC patients with higher serum miR-25 had short OS and DFS time. To further determine the possibility of miR-25 as an independent risk factor for prognosis, the level of miR-25 and other clinicopathological factors were evaluated by univariate and multivariate Cox regression analyses. Univariate Cox proportional hazards regression model revealed that low levels of miR-25, male, late-stage patients, tumour size >2cm, Lymph node metastasis and poorly differentiated tumours were independent predictors for OS, indicating that miR-25 may be one of novel molecular marker candidates for predicting the aggressive tumour development and poor prognosis of NSCLC. Multivariate Cox proportional hazards regression model revealed that TNM stage low levels of miR-25, tumour size, Lymph node metastasis were independent predictors for OS. Our results demonstrated that miR-25 is potential tumour biomarkers for the diagnosis of NSCLC. The ROC curve analyses revealed that miR-25 alone was useful as a tumour biomarker for the detection of NSCLC, with a high sensitivity and specificity.

A limitation is that, due to the case-control design of our study, blood samples were collected at the time of diagnosis. Also, our study lacks an external validation series. Despite the high accuracy and specificity of the qRT-PCR technique, miR-25 expression level measured can be influenced by both systematic experimental bias and technical variations including differences in sample procurement, stabilization, RNA extraction and sample differences.

In summary, the present study suggests that circulating miR-25 could serve as non-invasive diagnostic biomarkers for NSCLC. It could screen NSCLC from healthy population. However, the clinical application of miR-25 for lung cancer detection still needs further validation by future studies. This work represents an advance in biomedical science because it shows that serum miR-25 could discriminate NSCLC patients from healthy controls, so may represent a novel prognostic and diagnostic markers for NSCLC.

Summary table

What is known about this subject:

- A serum biomarker is needed for diagnosis and discrimination of NSCLC.
- Tissue miR-25 is significantly increased in NSCLC patients.

What this study adds:

- Serum miR-25 is significantly increased in NSCLC patients.
- Serum miR-25 is a novel serum prognostic and non-invasive diagnostic marker for NSCLC.

Disclosure statement

No potential conflict of interest was reported by the authors.

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