

Diagnostic and prognostic value of serum miR-145 and vascular endothelial growth factor in non-small cell lung cancer

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Abstract. Previous studies have reported the diagnostic and prognostic value of serum microRNA (miR)-145 and vascular endothelial growth factor (VEGF) levels in various types of cancer; however, their clinical use in non-small cell lung cancer (NSCLC) remains unclear. The present study included 215 patients, 106 with NSCLC and 109 with other lung diseases (OLDs). miR-145 expression levels were determined using reverse transcription-quantitative PCR (RT-qPCR) and VEGF levels were measured using an ELISA. The diagnostic performance was assessed using a receiver operating characteristic curve and area under the curve (AUC) analysis. A Kaplan-Meier survival curve and Cox regression analysis were employed to evaluate the prognostic significance of the markers. The biological function of miR-145 was examined in A549 and H1792 cell lines. The effects of miR-145 on cell proliferation of NSCLC cells were evaluated by flow cytometry, and the expression levels of miR-145 and cell cycle-related genes were determined by RT-qPCR. The results revealed that miR-145 and VEGF exhibited fair diagnostic performance [AUC, 0.61 (95% CI, 0.55-0.68) and AUC, 0.64 (95% CI, 0.57-0.71), respectively]. Combining age and smoking status with miR-145 and VEGF provided the best model for differentiating patients with NSCLC from those with OLDs (AUC, 0.76; 95% CI, 0.69-0.83). Furthermore, low serum miR-145

levels were associated with poor overall survival [hazard ratio (HR), 0.48; 95% CI, 0.27-0.85], whereas high VEGF levels were not associated with poor overall survival (HR, 1.47; 95% CI, 0.81-2.68). In addition, the results of the *in vitro* experiments indicated that miR-145 decreased cell proliferation via the induction of cell cycle arrest. In conclusion, the findings of the present study suggested that combining miR-145 and VEGF levels with clinical risk factors may be a potential diagnostic scheme for NSCLC. In addition, serum miR-145 may be used as a prognostic marker. These results indicated that miR-145 may function as a tumor suppressor in NSCLC.

Introduction

Lung cancer is a prevalent type of cancer worldwide, accounting for the highest number of new cases (11.4% of all cases) and deaths (18.0% of all deaths) in men in 2020 (1). Despite recent advances in treatment, and although numerous molecular signatures for diagnostic and prognostic purposes have been proposed, the incidence and mortality of lung cancer remain high. Increasing evidence has suggested that circulating microRNAs (miRNAs/miRs) and proteins in the blood may be dysregulated, and associated with cancer development and progression (2,3).

miRNAs, which are small single-stranded non-coding RNA molecules, serve important roles in various biological processes, such as cell proliferation, apoptosis, migration and invasion (4). miRNAs suppress gene expression at the post-transcriptional level by binding to the 3'-untranslated regions of mRNA (4). Among the miRNAs, miR-145 has been suggested to have a role in various types of cancer (5,6), including non-small cell lung cancer (NSCLC) (7,8). miR-145 exerts its functions by targeting specific genes, one of which is vascular endothelial growth factor (VEGF), a key regulator of angiogenesis (9). Zou *et al* (10) reported that miR-145 served an inhibitory role, both *in vitro* and *in vivo*, in the angiogenesis of breast cancer cells via VEGF-A regulation. Based on bioinformatics analysis in our previous study, VEGF was identified to be a downstream target gene of miR-145 (11). Therefore, the evaluation of both miR-145 and VEGF levels may provide further insight into their clinical use in NSCLC.

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Abbreviations: NSCLC, non-small cell lung cancer; miRNA, microRNA; VEGF, vascular endothelial growth factor; OLDs, other lung diseases; AUC, area under the curve; ROC, receiver operating characteristic; OS, overall survival

Key words: serum, miR-145, VEGF, diagnostic, prognostic, NSCLC

Previous studies have shown that serum miR-145 and VEGF levels may be associated with diagnosis and prognosis in glioblastoma (12), ovarian cancer (13,14) and NSCLC (15,16). Although there are an increasing number of studies that have supported the possibility of using serum miR-145 and VEGF as biomarkers for various purposes in lung cancer, the reports have been unclear regarding the prognostic role of these two markers (12,15-18). Furthermore, most of the studies have been conducted using healthy individuals as the control group, which could have led to an overestimation of the diagnostic accuracy (19).

The present study aimed to investigate the diagnostic performance of serum miR-145 and VEGF levels in patients with NSCLC and included patients with other lung diseases (OLDs) as the control group. The prognostic role of the two markers and the biological function of miR-145 in NSCLC cells were also evaluated.

Materials and methods

Study participants and blood samples. Clinical information on the study participants, as well as remaining samples, were obtained from our two previous studies following ethics approval from the Human Research Ethics Committee of the Faculty of Medicine, Prince of Songkla University (Songkhla, Thailand; approval nos. 59-011-05-1 and 60-350-04-2) (11,20). The participants were enrolled at Songklanagarind Hospital (Songkhla, Thailand) between January 2016 and December 2018. From the original 230 samples (117 NSCLC and 113 OLDs) of the previous study, 215 samples (106 NSCLC and 109 OLDs) were adequately obtained for the analysis of miR-145 expression and 183 samples (92 NSCLC and 91 OLDs) for VEGF determination. Patients with suspected lung cancer were included if they presented with a chronic cough for ≥ 8 weeks or hemoptysis more than once. In addition, information on smoking and drinking status, as well as the family history of cancer, for each participant were obtained by interviewing the patients. The demographic and clinical characteristics of the patients were retrieved from the hospital registry. Patients with a previous history of cancer or chemotherapy were excluded. During or after data collection, no personal information that could be used to identify individuals was available.

Confirmation of cancer was obtained via a pathological diagnosis. The histological type of NSCLC was diagnosed according to the 2015 World Health Organization classification of lung and pleural tumors (21). The clinical staging was based on the Tumor Node Metastasis staging system of the Cancer Staging Manual (7th Edition) of the American Joint Committee on Cancer (22). Patients with OLDs were diagnosed via routine procedures, including laboratory tests, chest X-rays and tissue biopsies. The mortality data were obtained from the civil registry.

Peripheral blood (5 ml) was collected at the time of diagnosis after the patients had provided their written informed consent, and samples were processed as previously described (11,20). Briefly, blood samples were coagulated for 30 min at room temperature and centrifuged at $3,400 \times g$ for 10 min at room temperature. Isolated serum was filtered through a polyvinylidene difluoride syringe filter with a pore

size of 0.22- μm (cat. no. SLGS033SB; MilliporeSigma) and prepared for miRNA isolation.

Cell culture. Human NSCLC cell lines, A549 and H1792 (both lung adenocarcinoma), were purchased from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium (cat. no. R6504; Sigma-Aldrich; Merck KGaA) supplemented with 10% (v/v) FBS (cat. no. 10091148; Gibco; Thermo Fisher Scientific, Inc.), and maintained at 37°C in a 5% CO_2 atmosphere.

miRNA transfection. miR-145 mimic (50 nM, 5'-GUC CAGUUUCCCCAGGAAUCCCU-3') and mimic negative control (NC) (50 nM, 5'-UCACAACCUCCUAGAAAGA GUAGA-3') were purchased from GE Healthcare Dharmacon, Inc. Transfection was conducted using Lipofectamine[®] 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, A549 (2×10^5) and H1792 (4×10^5) cells were suspended in medium containing 10% FBS and seeded into a T25 cell culture flask (Corning, Inc.) for 24 h before miRNA transfection. miR-145 mimic and mimic NC were diluted in Opti-MEM (cat. no. 31985088; Gibco; Thermo Fisher Scientific, Inc.), mixed with Lipofectamine 2000, and incubated for 30 min at room temperature. Subsequently, cells were incubated with the transfection mixture at 37°C in a 5% CO_2 atmosphere. Culture medium was replaced after 10 h, and the cells were further incubated at 37°C in a 5% CO_2 atmosphere for 4 days. After transfection, the cells were harvested and subjected to subsequent experiments. The efficiency of miRNA transfection was confirmed via reverse transcription-quantitative PCR (RT-qPCR). Untreated cells (cell group), cells treated with Opti-MEM and Lipofectamine 2000 (mock group) and cells transfected with mimic NC (mimic NC groups) were assigned as the control groups.

Detection of miR-145 expression using RT-qPCR. Total RNA was extracted from cells using TRIzol[®] reagent (cat. no. 15596018; Invitrogen; Thermo Fisher Scientific, Inc.) and from serum (250 μl) using a miRNeasy serum/plasma kit (cat. no. 217184; Qiagen GmbH), according to the manufacturers' protocols. miR-145 expression levels were measured using RT-qPCR, as previously described (11). Briefly, total RNA (50 ng) was reverse transcribed into cDNA using the miScript II RT kit (cat. no. 218161; Qiagen GmbH) at 37°C for 60 min and 95°C for 5 min using a thermal cycler (Bio-Rad Laboratories, Inc.). After dilution in 200 μl RNase-free water, miRNA amplification was performed using a miScript SYBR[®] Green PCR kit (cat. no. 218073; Qiagen, Inc.) on a CFX96 Touch Real-time qPCR Detection system (Bio-Rad Laboratories, Inc.). The miScript primer assay used for miR-145 and U6 small nuclear RNA 2 (RNU6-2, an internal control) was purchased from Qiagen, Inc. Mature miRNA sequences were used for designing the forward primers: miR-145, 5'-GUCCAGUUUCCCCAGGAAUCCCU-3' (cat. no. MS00003528; Qiagen GmbH) and RNU6-2, 5'-CGCTTCGGCAGCACATATACTA-3' (cat. no. MS00033740; Qiagen GmbH). The reverse primer was specifically designed for use with and contained in the miScript SYBR Green PCR kits (cat. no. 218073; Qiagen, Inc.).

Table I. Demographic and clinical characteristics, and miR-145 and VEGF levels among the study participants.

Variable	Number of patients (%)		P-value
	OLD (n=109)	NSCLC (n=106)	
Sex			0.030
Male	55 (50.5)	69 (65.1)	
Female	54 (49.5)	37 (34.9)	
Age, years			<0.001
<60	57 (52.3)	31 (29.2)	
≥60	52 (47.7)	75 (70.8)	
Smoking status			<0.001
Non-smoker	69 (63.3)	41 (38.7)	
Smoker	40 (36.7)	65 (61.3)	
Alcohol drinking			0.760
Non-drinker	67 (61.5)	63 (59.4)	
Drinker	42 (38.5)	43 (40.6)	
Family history of cancer			0.649
No	74 (67.9)	75 (70.8)	
Yes	35 (32.1)	31 (29.2)	
Histology			
ADE	-	77 (72.6)	
SCC	-	22 (20.8)	
Uncertain NSCLC	-	7 (6.6)	
Clinical stage			
I	-	9 (8.5)	
II	-	5 (4.7)	
III	-	12 (11.3)	
IV	-	80 (75.5)	
miR-145 expression			0.002
Low	20 (18.3)	39 (36.8)	
High	89 (81.7)	67 (63.2)	
VEGF level ^a			<0.001
Low	66 (72.5)	41 (44.6)	
High	25 (27.5)	51 (55.4)	

^aFewer patients (91 OLDs and 92 NSCLC) were used for the assessment of VEGF levels. ADC, adenocarcinoma; OLD, other lung disease; miR, microRNA; NSCLC, non-small cell lung carcinoma; SCC, squamous cell carcinoma; VEGF, vascular endothelial growth factor.

The qPCR amplification conditions were as follows: 95°C for 15 min for polymerase activation, followed by 50 cycles of denaturation at 94°C for 15 sec, amplification at 55°C for 30 sec and extension at 70°C for 30 sec. The difference between the cycle quantification (Cq) value of miR-145 and RNU6-2 (ΔCq) was calculated and the relative expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (23).

Detection of mRNA expression using RT-qPCR. Total RNA was extracted from the cells using TRIzol reagent, according to the manufacturer's protocol. To synthesize cDNA, 50 ng total RNA was reverse transcribed using iScript™ Reverse Transcription SuperMix (cat. no. 1708840; Bio-Rad Laboratories, Inc.) at 46°C for 20 min and 95°C for 1 min using a thermal cycler. qPCR was subsequently performed using a Luna® Universal

qPCR Master Mix (cat. no. M3003L; New England BioLabs, Inc.). The following thermocycling conditions were used for the amplification of CDK4, cyclin A, CDK1 and GAPDH: 95°C for 5 min for polymerase activation, followed by 40 cycles of denaturation at 95°C for 10 sec, amplification at 60°C for 30 sec and extension at 72°C for 30 sec. A CFX96 Touch Real-time qPCR Detection system was used for the analysis and the relative expression levels of the genes were calculated using the $2^{-\Delta\Delta Cq}$ method (24), using GAPDH as the endogenous control. The following primer sequences were used for qPCR: CDK4, forward 5'-GGAGGCCTTTGAACATCCCA-3' and reverse 5'-ACTGGCGCATCAGATCCTTA-3'; cyclin A, forward 5'-TAGACACCGGCAACTCAAG-3' and reverse 5'-TCTTCAGACTGGGAGAGGAGA-3'; CDK1, forward 5'-TGGAGAAGGTACCTATGGAGTTG-3' and reverse

Table II. Logistic regression analysis of predictors of the diagnosis of non-small cell lung carcinoma.

Variable	Crude OR (95% CI)	Adjusted OR (95% CI)	P-value
Sex			
Male vs. female	0.57 (0.32-1.03)		
Age			
≥60 vs. <60 years	2.43 (1.33-4.43)	2.16 (1.11-4.21)	0.023
Smoking status			
Smoker vs. non-smoker	2.38 (1.31-4.30)	2.92 (1.48-5.76)	0.002
Alcohol drinking			
Drinker vs. non-drinker	1.13 (0.62-2.04)		
Family history of cancer			
Yes vs. no	0.80 (0.43-1.50)		
miR-145 expression			
High vs. low	0.31 (0.15-0.61)	0.26 (0.12-0.57)	<0.001
VEGF level			
High vs. low	3.28 (1.77-6.09)	3.19 (1.64-6.20)	<0.001

miR, microRNA; OR, odds ratio; VEGF, vascular endothelial growth factor.

5'-AGGAACCCCTTCCTCTTCAC-3'; and GAPDH, forward, 5'-GACTTCAACAGCGACACCCACTCC-3' and reverse 5'-AGGTCCACCACCCTGTTGCTGTAG-3'.

ELISA. Serum VEGF levels were measured using a sandwich human ELISA kit (cat. no. 900-K10; PeprTech, Inc.) according to the manufacturer's instructions. Briefly, an ELISA plate was coated with 100 μ l capture antibody and incubated overnight at room temperature. After blocking with 1% BSA (cat. no. A9418; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, 100 μ l serum sample was added to each well, followed by 100 μ l HRP-conjugated detection antibody, after which the reaction was incubated for 1 h at room temperature. After washing, colorimetric detection was performed using an avidin-HRP conjugate (1:2,000) using ABTS liquid substrate solution (cat. no. 002024; Thermo Fisher Scientific, Inc.) for 10 min. Color development was measured at a wavelength of 405 nm, with a reference wavelength of 650 nm, using a microplate reader (Molecular Devices, LLC). VEGF levels were quantified using the constructed standard curve.

Cell viability assay. Cell suspension aliquots were mixed with an equal amount of 0.4% (w/v) trypan blue solution (cat. no. 15250061; Gibco; Thermo Fisher Scientific, Inc.) and the viable cells were counted under light microscopy using a hemocytometer. The number of cells in the cell group was considered as 100%, whereas the number of cells in the other groups was expressed as a percentage of that compared with the cell group.

In addition, a cell suspension was prepared at a concentration of 1×10^6 cells/ml in culture medium and a 50- μ l cell suspension was mixed with 450 μ l Muse[®] Count & Viability reagent (cat. no. 637365; MilliporeSigma). The mixed cells were incubated at room temperature in the dark for 5 min, then the live and dead cells were counted using a Muse cell

analyzer (MilliporeSigma). The number of total cells in the cell group was considered as 100%, whereas the number of total cells in the other groups was expressed as a percentage of that compared with the cell group. The percentage of live and dead cells in a population was determined.

Cell cycle analysis. After transfection, cells were trypsinized and centrifuged at 300 x g for 5 min at room temperature. Subsequently, 1×10^6 cells were fixed with ice-cold 70% methanol (cat. no. 67-56-1; RCI Labscan Ltd.) overnight at -20°C. Fixed cells were centrifuged at 300 x g for 5 min and washed with 500 μ l cold PBS. The cells were subsequently resuspended in 1 ml 1X binding buffer and a 100- μ l cell suspension (1×10^5 cells) was mixed with 5 μ l PI (cat. no. 556463; BD Biosciences) and 1 μ l RNase A, DNase and protease-free (10 mg/ml; cat. no. EN0531; Invitrogen; Thermo Fisher Scientific, Inc.). After incubation for 15 min at room temperature in the dark, 400 μ l 1X binding buffer was added to the cells, and the cell cycle distribution was analyzed using an Amnis[®] ImageStream[®]X Mk II and IDEAS software, version 6.0 (both Luminex Corporation). The percentage of cells distributed in each phase of the cell cycle was determined.

Statistical analysis. Statistical analysis was performed using R statistical software, version 3.4.4 (RStudio, Inc.) and GraphPad Prism 5.0 software (GraphPad Software, Inc.). The experiments were performed in triplicate. Clinical characteristics of the patients were presented as numbers and percentages. The continuous data of the cell viability assay and cell cycle analysis, which were obtained from three independent experiments, were presented as the mean \pm SD. Serum miR-145 and VEGF levels were presented as the median and interquartile range (IQR). The association between clinical variables and patient status (NSCLC and OLDs) was analyzed using a χ^2 test. The statistical differences between multiple groups were

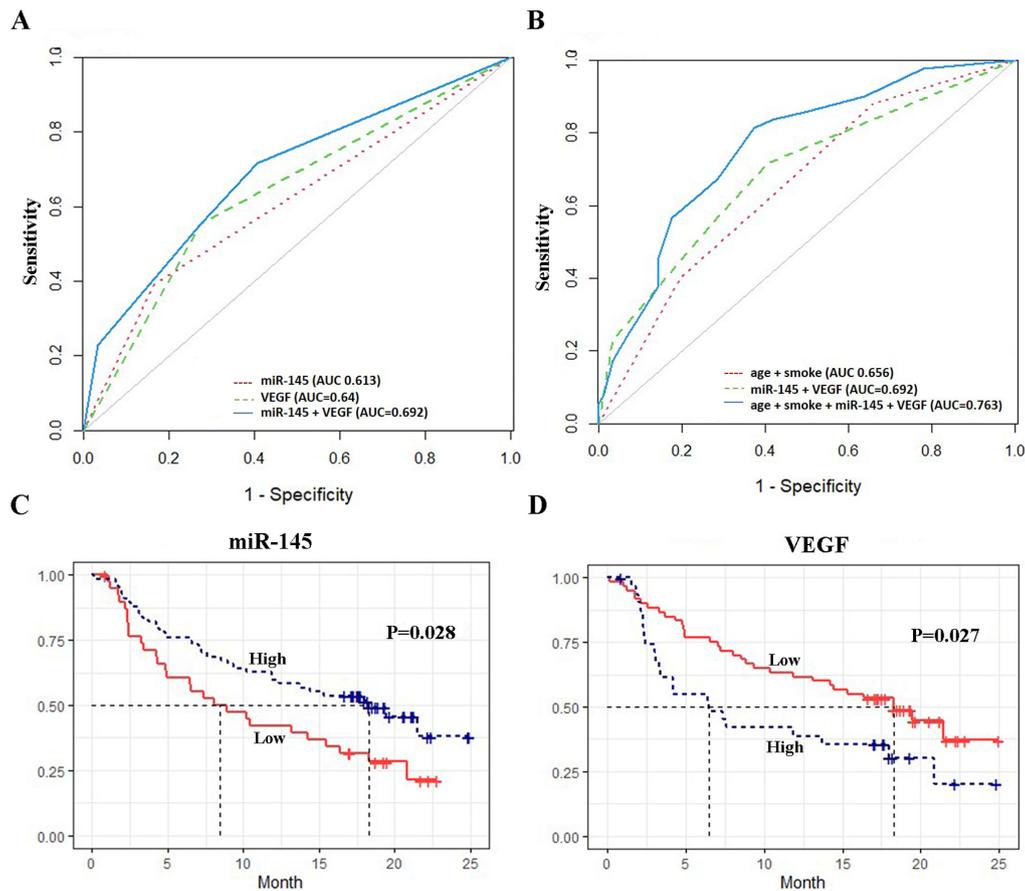


Figure 1. (A and B) Receiver operating characteristic curve analysis for the diagnosis of NSCLC. Kaplan-Meier curves of overall survival of patients with NSCLC according to (C) miR-145 expression and (D) VEGF levels. AUC, area under the curve; NSCLC, non-small cell lung cancer; miR, microRNA; VEGF, vascular endothelial growth factor.

determined using one-way ANOVA followed by Tukey's post hoc test. Logistic regression was used to evaluate the variables associated with the cancer diagnosis. The diagnostic performance was assessed using a receiver operating characteristic (ROC) curve and area under the curve (AUC) analysis, which was reported with 95% CIs. The cut-off values for miR-145 and VEGF were obtained from the best coordinate in the ROC to provide a maximum sum of sensitivity and specificity. For the survival analysis, a Kaplan-Meier curve was constructed and the statistical differences in overall survival (OS) among the various categories of variables were determined using a log-rank test. A multivariate Cox proportional hazards model was used to identify independent prognostic variables. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Demographic and clinical characteristics of the patients. The present study included 215 patients, 106 with NSCLC and 109 with OLDs. The diagnoses for the patients with OLDs were tuberculosis, bronchiectasis, interstitial lung disease, pneumonia or chronic obstructive pulmonary disease. Table I lists the clinicopathological characteristics of the patients, and shows the significant differences identified in sex ($P = 0.030$), age ($P < 0.001$) and smoking status ($P < 0.001$) between the NSCLC and control groups. There were no significant

differences identified between the two groups for alcohol consumption and family history of cancer.

Serum miR-145 and VEGF levels. The median serum miR-145 level was lower in the NSCLC group (17.15; IQR, 1.50-136.72) compared with that in the control group (25.46; IQR, 4.56-109.90). The median VEGF level was higher in the NSCLC group (0.04; IQR, 0.02-0.05) compared with that in the control group (0.02; IQR, 0.00-0.03). The cut-off values for miR-145 and VEGF levels were set as follows: ≤ 3.434 for miR-145 and ≤ 0.032 for VEGF for the lower value and > 0.434 for miR-145 and > 0.032 for VEGF for the upper value. A larger proportion of the NSCLC group had lower miR-145 levels compared with those in the control group (36.8 vs. 18.3%; $P = 0.002$; Table I). By contrast, a larger proportion of the NSCLC group had higher VEGF levels compared with those in the control group (55.4 vs. 27.5%; $P < 0.001$).

Diagnostic performance of serum miR-145 and VEGF. The logistic regression analysis revealed that smoking status, and miR-145 and VEGF levels were significant predictors for diagnosing NSCLC (Table II). Age also exhibited a moderate trend as a predictor ($P = 0.023$). Low miR-145 expression levels [adjusted odds ratio (aOR), 0.26; 95% CI, 0.12-0.57] and high VEGF expression levels (aOR, 3.19; 95% CI, 1.64-6.20) were associated with a NSCLC diagnosis.

Table III. Discriminative performance of miR-145, VEGF and clinical factors for the diagnosis of non-small cell lung carcinoma.

Variable	Diagnostic performance		
	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
miR-145	0.61 (0.55-0.68)	0.39 (0.29-0.49)	0.84 (0.76-0.91)
VEGF	0.64 (0.57-0.71)	0.55 (0.45-0.65)	0.73 (0.63-0.81)
miR-145 + VEGF	0.69 (0.62-0.76)	0.70 (0.27-0.80)	0.64 (0.52-0.97)
Age + Smoking + miR-145 + VEGF	0.76 (0.69-0.83)	0.80 (0.55-0.89)	0.66 (0.54-0.87)

AUC, area under the curve; miR, microRNA; VEGF, vascular endothelial growth factor.

Table IV. Cox regression analysis for overall survival in non-small cell lung carcinoma (n=106).

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Sex						
Male	Reference					
Female	0.67	0.39-1.16	0.152			
Age						
<60 years	Reference					
≥60 years	0.89	0.52-1.55	0.696			
Smoking status						
Non-smoker	Reference					
Smoker	1.56	0.92-2.64	0.097			
Drinking status						
Non-drinker	Reference					
Drinker	1.58	0.96-2.6	0.069			
Family history of cancer						
No	Reference					
Yes	0.70	0.39-1.26	0.237			
Staging						
I-II	Reference					
III-IV	3.63	1.31-10.06	0.013	3.13	1.10-8.90	0.013
Histologic type						
ADC	Reference					
SCC	1.13	0.62-2.07	0.694	1.13	0.62-2.07	0.693
Uncertain NSCLC	3.10	1.31-7.34	0.010	3.09	1.31-7.34	0.010
miR-145 expression						
Low	Reference					
High	0.58	0.35-0.95	0.030	0.48	0.27-0.85	0.012
VEGF level						
Low	Reference					
High	1.84	1.07-3.18	0.029	1.47	0.81-2.68	0.203

ADC, adenocarcinoma; HR, hazard ratio; miR, microRNA; NSCLC, non-small cell lung carcinoma; SCC, squamous cell carcinoma; VEGF, vascular endothelial growth factor.

The diagnostic power of miR-145 and VEGF, with or without other risk factors, was evaluated using ROC curve analysis. The AUC was 0.61 (95% CI, 0.55-0.68) for miR-145 alone and 0.64 (95% CI, 0.57-0.71) for VEGF alone. When

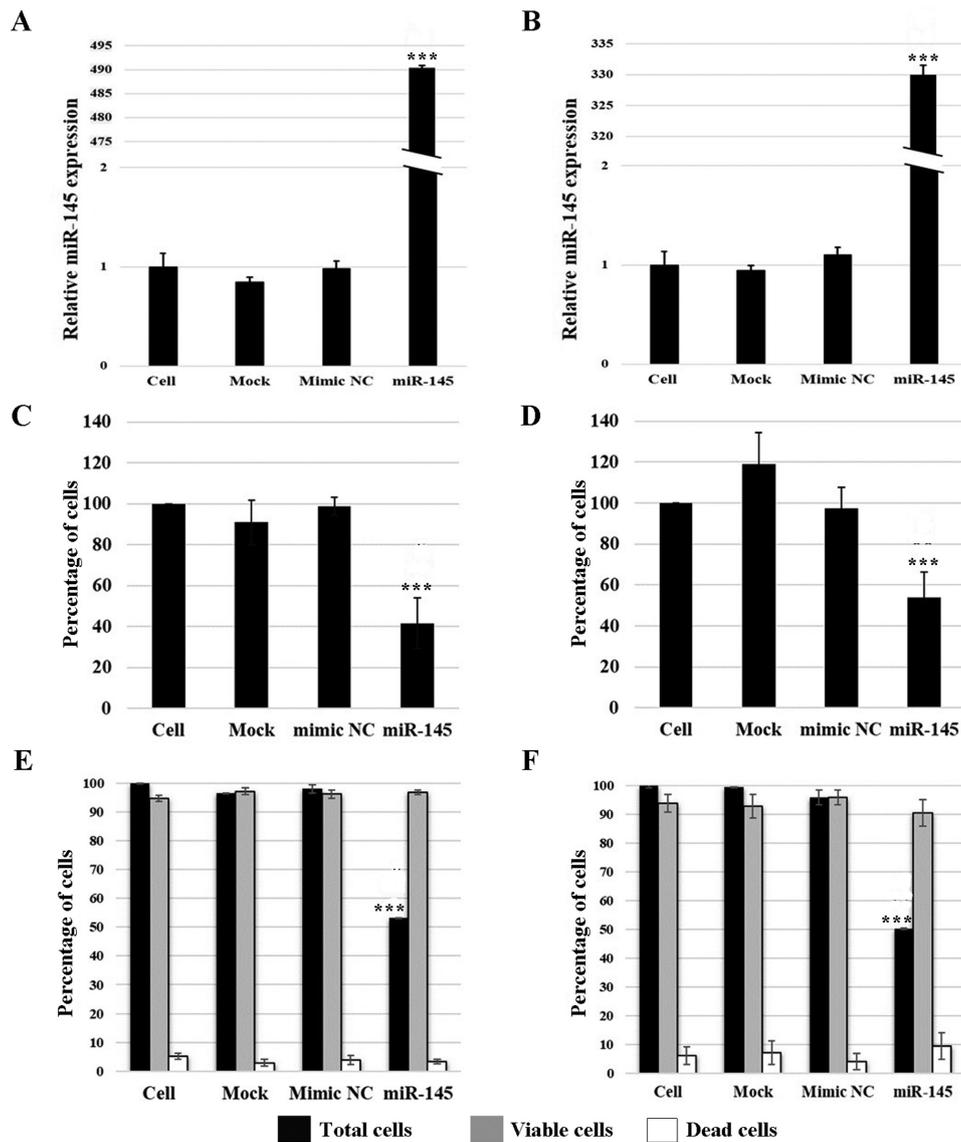


Figure 2. Reverse transcription-quantitative PCR analysis was performed to detect miR-145 expression in (A) A549 and (B) H1792 cells. Effect of upregulated miR-145 expression on viability was determined using a trypan blue assay in (C) A549 and (D) H1792 cells, and using a viability assay in (E) A549 and (F) H1792 cells. The cell group was set as 100%. All experiments were performed in triplicate on cell-culture passages 10-30. Data are presented as the mean \pm SD of three independent experiments. *** $P \leq 0.001$ vs. cell group. miR, microRNA; NC, negative control.

combined, miR-145 and VEGF improved the discrimination, with an AUC of 0.69 (95% CI, 0.62-0.76) (Fig. 1A). The combination of age, smoking status, miR-145 and VEGF expression proved to be the best model for differentiating patients with NSCLC from those with OLDs (AUC, 0.76; 95% CI, 0.69-0.83), with an optimal sensitivity and specificity of 80.4 and 65.9%, respectively (Table III; Fig. 1B).

Association between serum miR-145 and VEGF levels and OS. The patients had a median survival time of 14.24 months. The Kaplan-Meier curve showed that low miR-145 levels were associated with a significantly shorter OS ($P=0.028$), whereas low VEGF levels were associated with a longer OS ($P=0.027$) (Fig. 1C and D). Table IV shows the results of the multivariate Cox regression analysis. High miR-145 expression was independently associated with a higher OS [hazard ratio (HR), 0.48; 95% CI, 0.27-0.85]. By contrast, high VEGF levels were associated with a lower OS (HR, 1.47; 95% CI, 0.81-2.68);

however, the association was not statistically significant. Stage and histological types were also significantly associated with OS, whereas age, smoking status, drinking status and family history of cancer were not.

Effects of upregulated miR-145 expression on cell viability. The functional role of miR-145 in A549 and H1792 adenocarcinoma cells was further explored following its overexpression. Transfection success was confirmed by measuring miR-145 expression levels using RT-qPCR. The results revealed that miR-145 expression levels were upregulated by 494- and 330-fold in transfected A549 and H1792 cells, respectively, compared with the cell group ($P \leq 0.001$; Fig. 2A and B). The expression of miR-145 was not significantly different among the control groups.

As determined using the trypan blue assay, miR-145 overexpression resulted in a 58.5% reduction in total A549 cell numbers and a 42.9% reduction in H1792 cell numbers

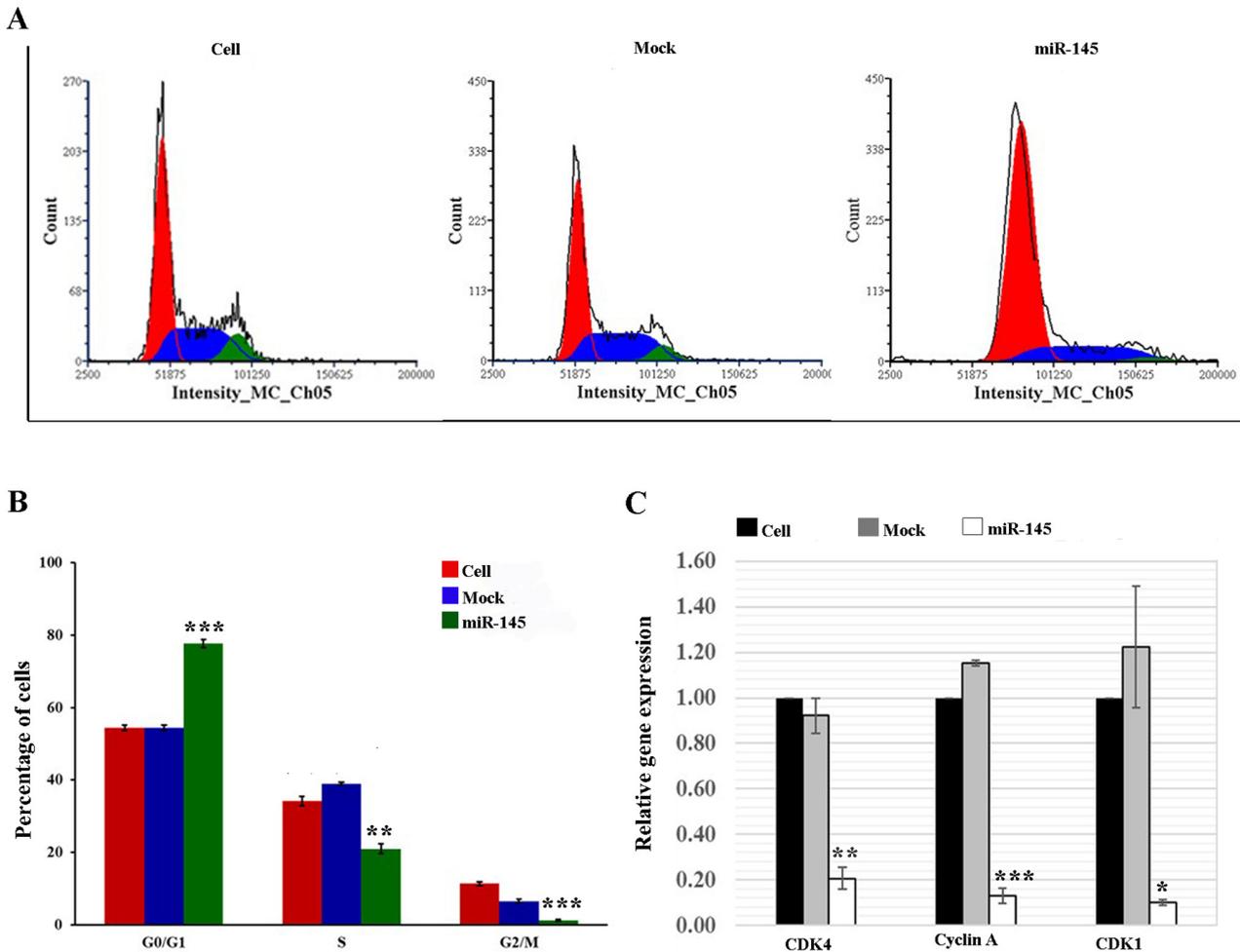


Figure 3. (A and B) Cell cycle progression was analyzed via flow cytometry in A549 cells. (C) Expression of cell cycle regulatory genes was determined using reverse transcription-quantitative PCR. All experiments were performed in triplicate on cell-culture passages 10-30. Data are presented as the mean \pm SD from three independent experiments. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$ vs. cell group. miR, microRNA; NC, negative control.

compared with the cell group ($P \leq 0.001$; Fig. 2C and D). Similarly, the results of the cell viability assay using the Muse cell analyzer revealed that the total cell numbers were significantly decreased following the overexpression of miR-145, by ~45.0% for A549 and 49.4% for H1792 cells compared with those in the cell group ($P \leq 0.001$; Fig. 2E and F). However, miR-145 overexpression had no effect on the number of viable and dead cells compared with in the control cells.

Effects of upregulated miR-145 expression on the cell cycle distribution. To further explore whether a reduction in total cell numbers following miR-145 overexpression was caused by cell cycle arrest, cell cycle analysis was performed using flow cytometry. Since there were no significant differences among the three control groups (data not shown), untreated cells and mock cells were used as the controls for subsequent experiments on the effects of miR-145 overexpression on cell cycle distribution and the expression of cell cycle regulatory genes. In A549 cells, the results revealed that the percentage of cells in G₀/G₁ phase was significantly increased by 42.7% ($P \leq 0.001$) in the miR-145 mimic-transfected cells compared with the cell group. Significant reductions in the number of cells in the S-phase (38.5%; $P \leq 0.01$) and G₂/M-phase (88.9%; $P \leq 0.001$) were also observed compared with the cell group (Fig. 3A

and B). By contrast, the miR-145 mimic-transfected H1792 cells exhibited an 18.1% decrease in the percentage of cells in G₀/G₁ phase compared with that in the cell group ($P \leq 0.05$), and a 36.4% increase in the percentage of cells in the S phase compared with in the cell group ($P \leq 0.05$; Fig. 4A and B).

Effects of upregulated miR-145 expression on cell cycle regulatory genes. In A549 cells, the expression levels of CDK4, cyclin A and CDK1 were significantly downregulated in the miR-145 mimic-transfected cells by 81.0 ($P \leq 0.01$), 88.6 ($P \leq 0.001$) and 91.5% ($P \leq 0.05$), respectively, compared with those in the cell group (Fig. 3C). Similar to the A549 cells, there was a significant reduction in CDK4 (76.1%; $P \leq 0.001$), cyclin A (91.7%; $P \leq 0.01$) and CDK1 (68.5%; $P \leq 0.05$) expression in the miR-145 mimic-transfected H1792 cells when compared with the cell group (Fig. 4C).

Discussion

The results of the present study demonstrated that miR-145 and VEGF may be useful in terms of their clinical use as diagnostic and prognostic biomarkers in NSCLC. The data revealed that combining the evaluation of patient clinical factors, including age and smoking status, with the measurement of serum

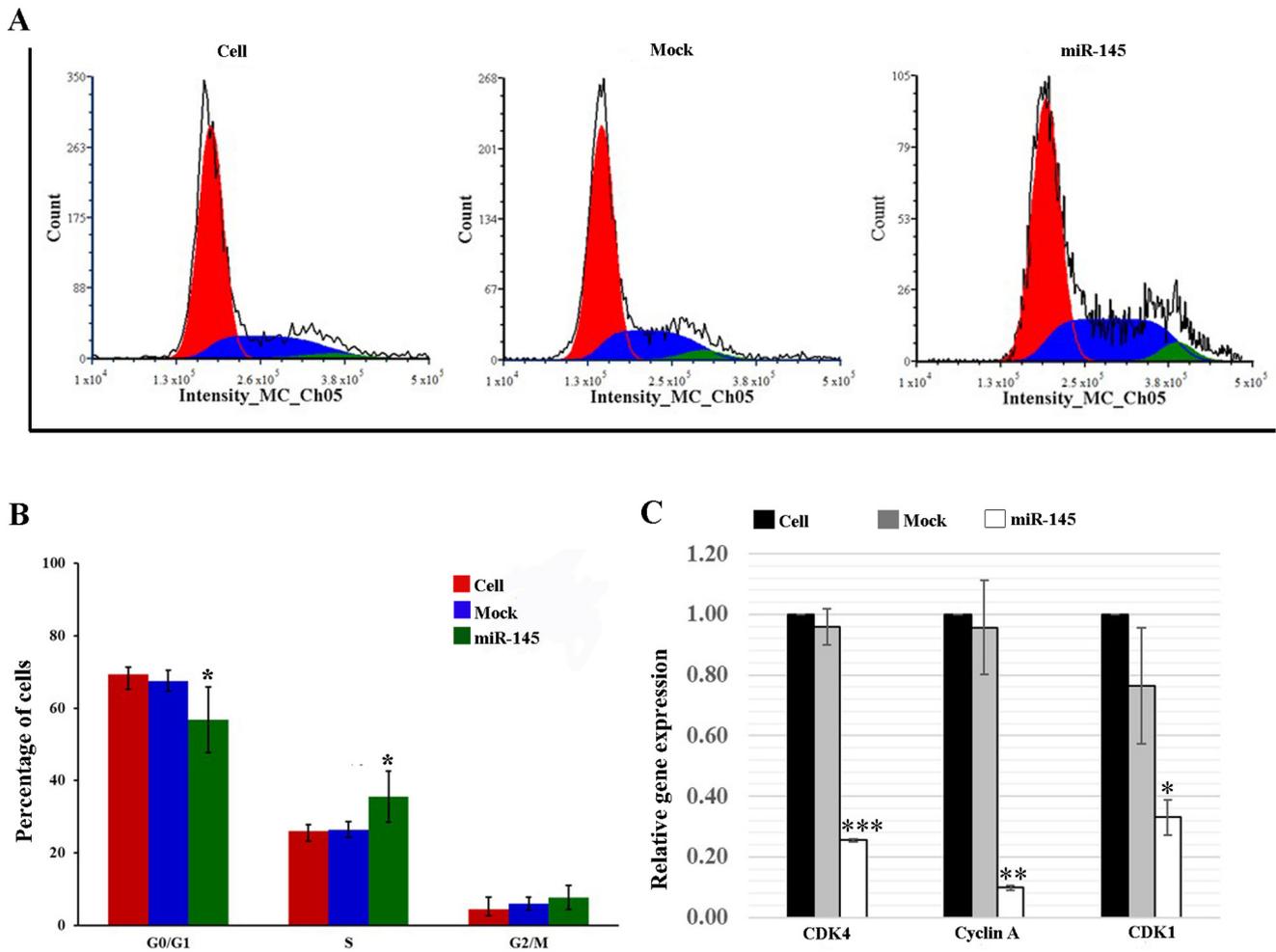


Figure 4. (A and B) Cell cycle progression was determined using flow cytometry in H1792 cells. (C) Expression of cell cycle regulatory genes was determined using reverse transcription-quantitative PCR. All experiments were performed in triplicate on cell-culture passages 10-30. Data are presented as the mean \pm SD from three independent experiments. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$ vs. cell group. miR, microRNA; NC, negative control.

miR-145 and VEGF levels was the best model for differentiating patients with NSCLC from those with OLDs, yielding an AUC of 0.76 with 80.4% sensitivity and 65.9% specificity. The results also revealed that downregulated miR-145 and upregulated VEGF levels were significantly associated with poor survival, and that miR-145 expression was an independent prognostic biomarker for NSCLC. The *in vitro* experimental results also revealed that miR-145 overexpression reduced cell viability via the induction of cell cycle arrest.

Serum miR-145 expression was significantly decreased in patients with NSCLC compared with that in the control group. miR-145 expression has also been shown to be reduced in the serum of patients with glioblastoma (12) and ovarian cancer (13) when compared with healthy controls. However, the results of the present study differ from those of Wang *et al* (15), which reported higher serum miR-145 levels in patients with NSCLC (n=70) compared with those in healthy controls (n=70). The discrepancy may be due to the difference in histological ratio. The study by Wang *et al* (15) included 48 patients with adenocarcinoma and 20 with squamous cell carcinoma, whereas the present study included 77 patients with adenocarcinoma and 22 patients with squamous cell carcinoma. Similar to serum miR-145 levels, the trend in serum VEGF levels in the current study was similar to those of other studies. Numerous studies

have reported higher serum VEGF levels in patients with cancer compared with in healthy individuals, including within breast (25), gastrointestinal (26), ovarian (14) and lung (27) cancer. Similarly, Zhang *et al* (27) revealed that VEGF levels were upregulated in patients with lung cancer compared with those with tuberculosis.

For diagnostic evaluations, the present study results demonstrated that the AUC of serum miR-145 was 0.61. By contrast, a study by Wang *et al* (15) indicated that serum miR-145 was a good biomarker for differentiating patients with NSCLC from healthy controls, with an AUC of 0.84 (15). The AUC for VEGF in the present study was 0.64, which is similar to that measured in a study by Chakra *et al* (16), which reported an AUC value of 0.66 (16). The conflicting findings between the present results and those of the study by Wang *et al* (15) may be due to the use of a different control; the present study enrolled patients with OLDs as the control group, whereas the study by Wang *et al* included healthy individuals as the control group.

In the present study, clinical risk factors (age and smoking status) were measured alongside miR-145 and VEGF, and an increased AUC value was observed for the predictive model (AUC, 0.76), which is consistent with the findings of other studies (20,28). For example, in a previous study, the combination of 24 miRNAs with major lung cancer risk factors, such

as sex, age and smoking status, was discovered to improve the AUC to 0.94 compared with using the risk factors in isolation (AUC, 0.72) (28). Similar to the present study, our previous study showed that the AUC value of the combined clinical factors and miR-339-3p was increased to 0.71 compared with the AUC of miR-339-3p alone, which was 0.62 (20). This result suggested that a combination of biomarkers and risk factors may provide valuable insights for predictive accuracy.

In terms of the prognostic role, the findings of the present study revealed that low serum miR-145 and high VEGF levels were associated with poor survival in NSCLC. Zhang *et al* (12) revealed that patients with glioblastoma with low serum miR-145 levels had a shorter survival compared with those with high miR-145 levels. Similarly, Liang *et al* (13) demonstrated that low miR-145 expression was associated with the prognosis of ovarian cancer. These results indicated that miR-145 may be involved in lung tumorigenesis and may serve as an invasive prognostic biomarker for NSCLC. For VEGF, the findings of the present study identified no significant role as an independent prognostic biomarker for NSCLC. Previous studies have indicated that high serum VEGF levels may be an independent prognostic factor in ovarian (14) and gastrointestinal cancer (26). In lung cancer, two studies have indicated that high VEGF levels were useful for predicting a shorter OS (16,29). By contrast, a study by Akin Kabalak *et al* (18) showed that serum VEGF levels did not significantly predict the prognosis of lung cancer, either in NSCLC or small cell lung cancer (18). Similarly, Zhang *et al* (27) found no differences in OS between patients with high and low serum VEGF levels in lung cancer. The results of the current study are consistent with those of the two latter studies, suggesting that high VEGF levels were not an independent prognostic biomarker. The small number of patients with NSCLC investigated in the present study may be one of the reasons for this lack of significance.

In the present study, the biological function of miR-145 in NSCLC was also examined. The results revealed that miR-145 led to a decrease in cell number, with no effect on cell death. Furthermore, miR-145 overexpression induced cell cycle arrest at the G0/G1-phase for A549 cells and at the S-phase for H1792 cells. This may indicate that miR-145 affected the cell cycle at different phases depending on the cell type. In addition, the present results revealed that miR-145 inhibited the cell cycle progression by modulating the expression of cell cycle regulators, including CDK1, CDK4 and cyclin A, in both A549 and H1792 cells. These results are consistent with previous studies from other groups, which reported that miR-145 prevented cell proliferation in gastric cancer (30), hepatocellular carcinoma (31) and NSCLC (32-34). In addition, previous studies have shown that increased miR-145 expression inhibited invasion and metastasis in numerous types of cancer, including osteosarcoma (35), hepatocellular carcinoma (31), nasopharyngeal carcinoma (36) and lung cancer (8). Taken together, the findings of the present study suggested that miR-145 may serve a crucial role in NSCLC as a tumor suppressor miRNA.

Nevertheless, there are several limitations to the current study that should be addressed in future research. Firstly, transcript levels only are not sufficient to predict protein levels; however, due to budget restrictions, only the alterations in gene expression levels were detected. Cell cycle analysis was

performed and gene expression of related cell cycle regulators was determined, and the results suggested a potential role of miR-145 in cell cycle regulation. Regarding the clinical implications of miR-145 and VEGF, the ideal cancer diagnostic biomarker would be most beneficial if the cancer could be detected as early as possible. This type of study should include a substantial proportion of early-stage disease; however, in the present study, >80% of the patients had late-stage disease, which may lead to an overestimated diagnostic power. However, OLDs were included as control subjects rather than healthy individuals, as this may have caused an overestimation of the diagnostic performance. Therefore, using the current study design, the resultant diagnostic power estimation, to a certain extent, was balanced.

In conclusion, the findings of the present study demonstrated that patients with NSCLC had lower serum miR-145 expression levels and higher serum VEGF levels compared with those in patients with OLDs. Serum miR-145 expression was identified to be a useful diagnostic and prognostic marker in NSCLC, whereas serum VEGF was only discovered to have potential as a diagnostic biomarker. The present study, which was based on findings obtained from clinical samples and *in vitro* experiments, suggested a role for miR-145 as a tumor suppressor in NSCLC. In addition, to the best of our knowledge, the present study led to the innovative finding that combined evaluation of miR-145 and VEGF enhanced the diagnostic performance of lung cancer diagnosis. However, further studies regarding the molecular target and specific biological pathway of miR-145 are warranted.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PR, PT and SLG conceived and designed the study. PR, PC and KT developed and designed the methodology. SLG and WK acquired the clinical data and managed the patients. PR, SB and PT performed the statistical analysis and interpreted the data. PR and PT supervised the study. PR and PT confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments were approved by the Human Research Ethics Committee of the Faculty of Medicine, Prince of Songkla

University (approval nos. 59-011-05-1 and 60-350-04-2). Patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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