

RESEARCH ARTICLE

Serum miRNAs associated with tumor-promoting cytokines in non-small cell lung cancer

Pichitpon Chaniad¹, Keson Trakunran¹, Sarayut Lucien Geater², Warangkana Keeratichananont², Paramee Thongsuksai³, Pritsana Raungrut^{1*}

1 Department of Biomedical Science, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand, **2** Division of Respiratory and Respiratory Critical Care Medicine, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand, **3** Department of Pathology Department, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand

* rpritsan@medicine.psu.ac.th



OPEN ACCESS

Citation: Chaniad P, Trakunran K, Geater SL, Keeratichananont W, Thongsuksai P, Raungrut P (2020) Serum miRNAs associated with tumor-promoting cytokines in non-small cell lung cancer. PLoS ONE 15(10): e0241593. <https://doi.org/10.1371/journal.pone.0241593>

Editor: Esra Bozgeyik, Tekirdag Namik Kemal University, TURKEY

Received: March 12, 2020

Accepted: October 16, 2020

Published: October 30, 2020

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pone.0241593>

Copyright: © 2020 Chaniad et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its [Supporting information](#) files.

Abstract

Tumor-promoting cytokines are a cause of tumor progression; therefore, identifying key regulatory microRNAs (miRNAs) for controlling their production is important. The aim of this study is to identify promising miRNAs associated with tumor-promoting cytokines in non-small cell lung cancer (NSCLC). We identified circulating miRNAs from 16 published miRNA profiles. The selected miRNAs were validated in the serum of 32 NSCLC patients and compared with 33 patients with other lung diseases and 23 healthy persons using quantitative real-time PCR. The cytokine concentration was investigated using the enzyme-linked immunoassay in the same sample set, with clinical validation of the miRNAs. The correlation between miRNA expression and cytokine concentration was evaluated by Spearman's rank correlation. For consistent direction, one up-regulated miRNA (miR-145) was found in four studies, and seven miRNAs were reported in three studies. One miRNA (miR-20a) and four miRNAs (miR-25-3p, miR-223, let-7f, and miR-20b) were reported in six and five studies. However, their expression was inconsistent. In the clinical validation, serum miR-145 was significantly down-regulated, whereas serum miR-20a was significantly up-regulated in NSCLC, compared with controls. Regarding serum cytokine, all cytokines [vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and transforming growth factor β (TGF- β)], except tumor necrosis factor- α (TNF- α), had a higher level in NSCLC patients than controls. In addition, we found a moderate correlation between the TGF- β concentration and miR-20a ($r = -0.537$, $p = 0.002$) and miR-223 ($r = 0.428$, $p = 0.015$) and a weak correlation between the VEGF concentration with miR-20a ($r = 0.376$, $p = 0.037$) and miR-223 ($r = -0.355$, $p = 0.046$). MiR-145 and miR-20a are potential biomarkers for NSCLC. In addition, the regulation of tumor-promoting cytokine, through miR-20a and miR-223, might be a new therapeutic approach for lung cancer.

Funding: This study is funded by a grant from the Prince of Songkla University [grant number MED590692S], the Research Center for Cancer Control in Thailand [grant number MEDRC59036], and the Faculty of Medicine, Prince of Songkla University [grant number REC60350042]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Lung cancer is the most common and leading cause of cancer death worldwide, accounting for 11.6% of the total cases and 18.4% of the total cancer deaths in 2018, respectively [1]. Approximately 84% of lung cancer is non-small cell lung cancer (NSCLC), which is usually diagnosed in the advanced stage (30%–79% of all NSCLC cases) and accompanied by lymph node and distant metastasis [2, 3]. Tumor progression is supported by tumor-promoting cytokines, in which high serum levels of several cytokines, such as vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), transforming growth factor β (TGF- β), and tumor necrosis factor- α (TNF- α), are correlated with advanced stages of lung cancer [4, 5]. Currently, there is accumulating evidence showing that these cytokines are regulated by microRNAs (miRNAs). Therefore, the identification of miRNAs associated with cytokine production is desired.

MiRNAs are short noncoding RNAs, 20–22 nucleotides in length. They suppress gene expression at the post-transcriptional level by binding to the 3'-untranslated regions of mRNAs [6]. Numerous studies have shown that miRNAs play a part in cell differentiation, proliferation, apoptosis, and cytokine production [6, 7]. In addition, the remarkable stability of serum miRNAs under various conditions was found in our previous report [8]. Several studies have revealed that the expression levels display oscillatory changes in response to TNF- α in macrophage cells [9], and VEGF and IL-6 in bone marrow mesenchymal stromal cells [10]. Moreover, changes in miRNA expression have been demonstrated to be involved with cytokine signaling pathways in several types of cancers, including colorectal [11], breast [12], and lung [13] cancers. Although there has been accumulating evidence to support the regulation of cytokines by miRNAs, studies conducted to determine the association of miRNAs and tumor-promoting cytokines in clinical samples in lung cancer are limited.

In this study, we identified differentially expressed (DE) miRNAs through the systematic review of previously published studies on miRNA profiling in NSCLC. A vote-counting procedure and bioinformatics analysis were used to obtain miRNAs associated with cytokine signaling pathways. We then validated the selected miRNAs using quantitative real-time PCR in serum samples of NSCLC compared with controls, which included patients with other lung diseases (OL) and healthy persons (HP). We also determined the serum concentration of the common tumor-promoting cytokines (IL-6, VEGF, TGF- β , and TNF- α) using the enzyme-linked immunoassay (ELISA) in samples from the same patients with miRNA validation. Finally, we investigated the association of miRNA expression and cytokine concentration in the serum of NSCLC patients.

Materials and methods

Literature search

The literature on miRNA profiling of lung cancer, published between 2006 and 2016, was retrieved from “PubMed” and “Scopus” databases. The search terms “miRNA or miR or microRNA,” “lung cancer,” and “profiling” were used.

Study selection criteria

Eligible studies were required to meet the following inclusion criteria: 1) miRNA expression profiling of patients with NSCLC; 2) conducted on blood, serum, or plasma; 3) used samples from patients with OL or HP for comparison; 4) were full-text articles in English. The exclusion criteria were: 1) patients received any previous treatment; 2) studies that did not report *p*-values, or reported a false discovery rate (FDR); 3) studies were review articles; 4) studies that did not report sample size.

Data extraction

Lists of DE miRNAs were extracted from each selected article. Related information was also retrieved, including authors, year of publication, country, specimen type, histological subtypes, number of samples (both cases and controls), stage of cancer, array platforms, cut-off criteria (p -value or FDR), and numbers of DE miRNAs. All articles were independently assessed by two researchers (Chaniad P and Raungrut P).

Ranking

The vote-counting strategy reported in Griffith's and Chan's studies [14, 15] was used. The DE miRNAs were ranked for importance in the following order: 1) the number of articles that consistently reported as differentially expressed; 2) direction of change of DE miRNA, and 3) the total number of samples. Moreover, we considered the number of target genes supported by reliable and experimental evidence from the miRTarBase 7.0 [16].

Bioinformatics analysis

The miRTarbase 7.0 containing experimentally validated miRNA-target interactions was used to identify potential gene targets [16]. Enrichment analyses of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were analyzed by DIANA-miRPath v3.0. [17]. The integrated genes regulatory network was generated by Cytoscape 3.5.1 [18]. Cytokine-related genes were retrieved from the Mouse Genome Informatics Web Site [19].

Study subjects and sample collection

This study was approved by the Ethics Committee on Human Research, Faculty of Medicine, Prince of Songkla University (REC 59-210-04-2 and REC 59-211-04-2). All subjects, including 32 NSCLC patients, 33 OL patients, and 23 HP, were recruited from Songklanagarind Hospital, Songkhla, Thailand, from May 2016 to April 2017. NSCLC was diagnosed according to 2015 WHO classification of lung and pleural tumors [20]. The staging of cancer was based on the Tumor Node Metastasis cancer staging system of the AJCC Cancer Staging Manual (7th Edition) [21]. No patients received any treatment before sample collection.

For the control groups, subjects who were age- and gender-matched with the NSCLC group were included. The OL group were patients who had symptoms similar to lung cancer, such as tuberculosis, bronchiectasis, interstitial lung disease, pneumonia, and chronic obstructive pulmonary disease. The HP group was recruited from people who had an annual checkup for two consecutive years. Written informed consent was obtained from all subjects. Peripheral blood (5 ml) was collected in a clotting tube (Greiner Bio-One, Kremsmünster, Austria). Serum was isolated by centrifugation and prepared for miRNA isolation, as previously described [8].

Quantitative real-time PCR (qRT-PCR)

The total RNA from serum was extracted using Trizol[®] LS (Invitrogen, California, USA) according to the manufacturer's protocol. The quantity and quality of the total RNA were measured using a NanoDrop[®] ND-1000 UV-Vis spectrophotometer (Thermo Scientific, Massachusetts, USA) at an optical density ratio of A260/280 nm and A260/230 nm. The total RNA (50 ng) was reverse transcribed into complementary DNA (cDNA) by the miScript II RT kit (Qiagen, Hilden, Germany). Reverse transcription was performed by Thermal cycler (Bio-Rad, California, USA) at 37 °C for 60 mins, and 95 °C for 5 mins. The cDNA was diluted by

adding 200 μ l of RNase-free water. Subsequently, miRNA amplification was performed using a Bio-Rad CFX96 qPCR system (Bio-Rad, California, USA) with the miScript SYBR[®] green PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Primers for miR-145-5p, miR-206-5p, miR-20a-5p, miR-223-5p, miR-25-3p, let-7f-5p, miR-20b-5p, and U6 small nuclear RNA (RNU6), were used as internal controls, and obtained from Qiagen (Qiagen, Hilden, Germany). Data were presented as the cycle threshold (Ct) value, which was determined using the default threshold settings. The difference between the Ct value (Δ Ct) of the miRNA of interest and RNU6 was calculated, and the relative expression was presented using the $2^{-\Delta\text{CT}}$ method [22].

Enzyme-linked immunoassay

Concentrations of VEGF, IL-6, TGF- β , and TNF- α were measured quantitatively using 100 μ l of serum with a sandwich human ELISA kit (Preprotech, Rehovot, Israel) according to the manufacturer's instructions. Absorbance was read at 405 nm with a reference wavelength of 650 nm by a microplate reader (Molecular Devices, California, USA). Samples were assayed in duplicate, and concentrations of each cytokine were calculated by the constructed standard curve.

Statistical analysis

Data distribution of relative miRNA expression and cytokine concentration was assessed by the Shapiro–Wilk normality test. Outliers of the data were detected using the Grubbs' test. Differences in the data between the groups of subjects were analyzed by the Student's *t*-test if the data had a normal distribution, and by the Wilcoxon–Mann–Whitney test in cases of non-normal distribution. The correlation between miRNA expression and cytokine concentration was analyzed by the Spearman's rank correlation. A *p*-value of less than 0.05 was considered statistically significant. All plots and statistical analyses were performed using the R-statistical software version 3.4.4.

Results

Included articles

[Fig 1](#) depicts the number of selected articles from each selection process. A total of 725 studies were identified using our search terms. There were 536 remaining articles, after removing 189 duplicates. After screening the titles and abstracts, 47 eligible articles were included. According to the exclusion criteria, only 16 articles were finally included in our analysis ([Fig 1](#)). All characteristics of these articles are shown in [Table 1](#).

Differentially expressed miRNAs

In total, 229 DE miRNAs were obtained from the 16 miRNA profiles studied, of which 181 and 48 miRNAs were reported with consistent and inconsistent direction (both up- and down-regulation), respectively. Among the 181 consistent miRNAs, 97 miRNAs were up-regulated, and 84 miRNAs were down-regulated.

Only miR-145 was reported to be consistently up-regulated in four studies. Six miRNAs (miR-320, miR-151a, miR-16, miR-200b-3p, miR-205, and miR-574) and one miRNA (miR-1285-3p) were most consistently reported to be up-regulated and down-regulated in three studies, respectively ([Table 2](#)). For inconsistent direction, miR-20a was reported in six studies (4 up-/2 down-regulated expression), and miRNAs (miR-25-3p, miR-223, let-7f, and miR-20b) were reported in five studies.

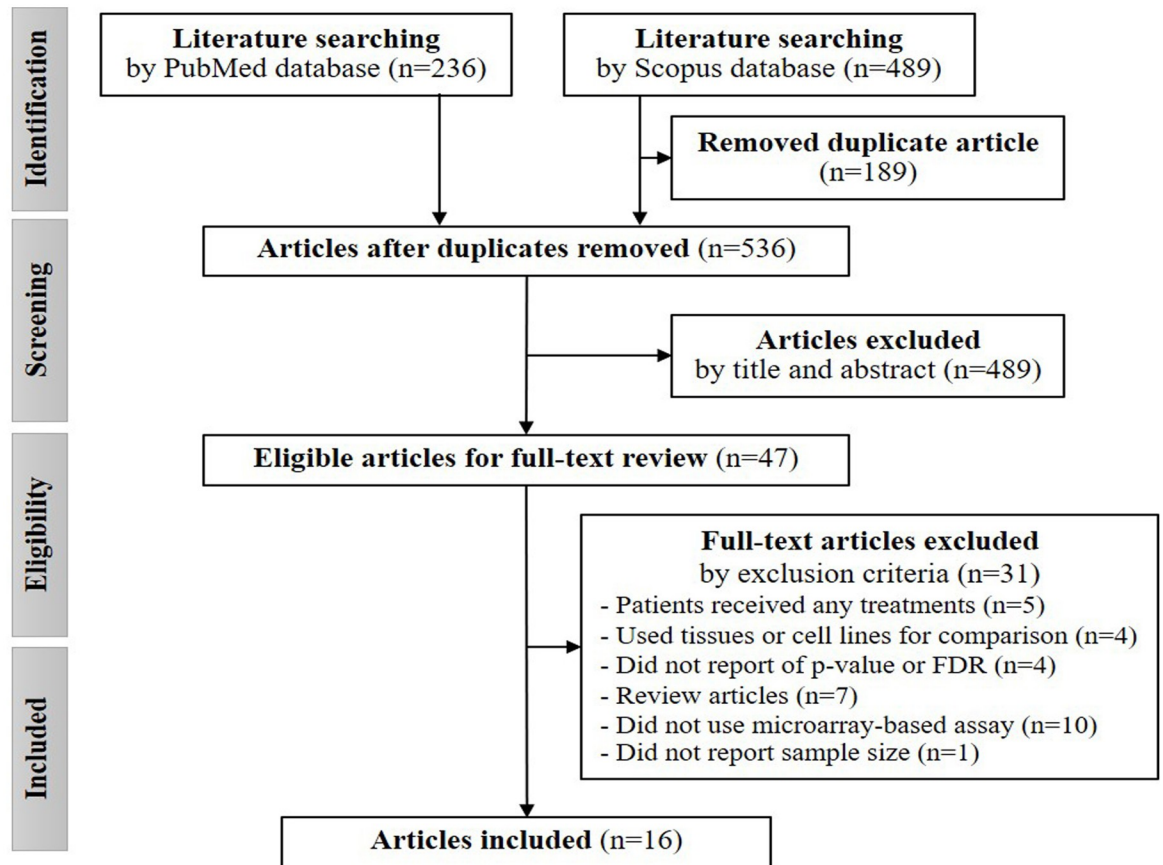


Fig 1. The study selection process.

<https://doi.org/10.1371/journal.pone.0241593.g001>

List of potential total genes and targeted genes that are supported by strong and experimental evidences was presented in [S1 Table](#) for the consistently up-regulated miRNA, [S2 Table](#) for the consistently down-regulated miRNA, and [S3 Table](#) for the inconsistency reported miRNA.

Functional enrichment analysis and gene interaction network

We performed a functional enrichment analysis to find the molecular networks and their target genes of seven DE miRNAs, including miR-145, miR-206, miR-20a, miR-223, miR-25-3p, let-7f, and miR-20b. The most significantly enriched GO terms on molecular functions, cellular components, and biological functions are iron binding (1662 genes), organelles (2973 genes), and cellular nitrogen metabolic processes (1499 genes) ([Fig 2A](#)). Also, the KEGG pathway showed that target genes were enriched in pathways for cancer (155 genes), PI3K-Akt signaling pathways (110 genes), proteoglycans in cancer (97 genes), MAPK signaling pathways (88 genes), and protein processing in the endoplasmic reticulum ([Fig 2B](#)). The complexity of the miRNA-mRNA interaction network is shown in [Fig 2C](#). The results showed that a single miRNA regulates numerous genes. The highest number of target genes were identified for miR-145 (143 genes), followed by miR-20a (71 genes). In addition, these miRNAs work synergistically. We also revealed that our selected miRNAs regulated cytokine-associated genes, such as VEGFA, IGF1R, TGFBR1, and HIF1A.

Table 1. Characteristics of included studies.

Study	Year	Country	Specimen type	Histological subtype	No. of sample (case/control)	Stage	Platform	Cut-off criteria	DE miRNA		
									Up	Down	Total
Fan <i>et al.</i> [23]	2016	China	Serum	NSCLC	152 (94/HP 58)	II, IIA-IIIB	Fluorescent coding liquid beads array (Shanghai Tellgen Life Science)	$p < 0.01$	6	1	7
Gao <i>et al.</i> [24]	2016	China	Plasma	SCC	10 (5/HP 5)	I	Taqman low-density array (Applied Biosystems)	$p < 0.01$	12	6	18
Rita <i>et al.</i> [25]	2016	Norway	Serum	ADC	70 (38/HP 16, COPD 16)	I-IV	Taqman low-density array (Applied Biosystems)	FDR < 0.01	10	27	37
Leidinger <i>et al.</i> [26]	2015	Germany	Blood	NSCLC	94 (74/HP 20)	I-IV	96.96 Dynamic array (Fluidigm)	$p < 0.05$	15	16	31
					100 (74/COPD 26)				10	21	31
Nadal <i>et al.</i> [27]	2015	American	Serum	NSCLC	92 (70/HP 22)	I-III	mirVana bioarrays v2.0 (Ambion)	$p \leq 0.001$	60	31	91
Wozniak <i>et al.</i> [28]	2015	Russia	Plasma	NSCLC	200 (100/HP 100)	IA-IIIA	TaqMan Human MicroRNA Array A + B Card Set v3.0 (Applied Biosystems)	$p < 0.05$	33	28	61
Geng <i>et al.</i> [29]	2014	China	Plasma	NSCLC	50 (25/HP 25)	I-II	microarray (Qiagen)	$p < 0.05$	12	0	12
Rani <i>et al.</i> [30]	2013	Ireland	Serum	ADC	80 (40/HP 40)	I-IV	Taqman low-density array (Applied Biosystems)	$p < 0.05$	6	2	8
Heegaard <i>et al.</i> [31]	2012	Denmark	Serum	NSCLC	440 (220/HP 220)	I + IA-IIIB	96.96 Dynamic array (Fluidigm)	$p < 0.05$	1	7	8
Patnaik <i>et al.</i> [32]	2012	American	Blood	ADC	45 (22/HP 23)	IA-IIIB	miRCURY locked nucleic acid microarrays (Exiqon)	FDR < 0.01	12	12	24
Roth <i>et al.</i> [33]	2012	Germany	Serum	NSCLC	32 (21/HP 11)	I-IV	Microfluid biochips (Febit Biomed GmbH)	$p < 0.05$	18	12	30
Chen <i>et al.</i> [34]	2012	China	Serum	NSCLC	310 (200/HP 110)	I-IV	Taqman probe-based qRT-PCR assay (Applied Biosystems)	$p < 0.05$	10	0	10
Foss <i>et al.</i> [35]	2011	America	Serum	NSCLC	22 (11/HP 11)	I-II	GenoExplorer microRNA Expression System (GenoSensor Corporation)	$p < 0.05$	8	0	8
Silva <i>et al.</i> [36]	2011	Spain	Plasma	NSCLC	48 (28/HP 20)	I-IV	Taqman low-density array (Applied Biosystems)	$p < 0.05$	0	10	10
Wang <i>et al.</i> [37]	2011	China	Serum	NSCLC	10 (5/HP 5)	I-III	Microarray chip (LC sciences)	FDR < 0.05	11	8	19
Keller <i>et al.</i> [38]	2009	Germany	Blood	NSCLC	36 (17/HP 19)	I-IV	Geniom Biochip miRNA homo sapiens (Febit Biomed GmbH)	$p < 0.05$	13	14	27

NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; SCC, squamous cell carcinoma; HP, healthy person; COPD, chronic obstructive pulmonary disease; FDR, false discovery rate; DE miRNA, differentially expressed miRNA.

<https://doi.org/10.1371/journal.pone.0241593.t001>

Validation of selected differentially expressed miRNAs

The expression level of seven miRNAs, including miR-145, miR-206, miR-20a, miR-223, miR-25-3p, let-7f, and miR-20b, was validated the serum of 32 patients with NSCLC, 33 patients with OL, and 23 HP using qRT-PCR analysis. NSCLC patients had a mean age of 59.9 years. Eighteen cases were male, and 14 were female. Histological subtype was 84.4% (27/32 of cases) for adenocarcinoma (ADC) and 15.6% (5/32 of cases) for squamous cell carcinoma (SCC). The majority of patients exhibited stage IV lung cancer (90.6%, 29/32 of cases), whereas one and two cases were stage II and I lung cancer, respectively. The OL group included 22 patients with chronic obstructive pulmonary disease, 6 with bronchiectasis, 3 with tuberculosis, 1 with pneumonia, and 1 with idiopathic pulmonary fibrosis.

Table 2. Differentially expressed miRNAs according to the ranking criteria.

miRNA	Mature sequence	Studies	Total sample (Case/Control)	Reference
Consistently up-regulated miRNA				
miR-145	16 GUCCAGUUUCCCAGGAAUCCCU 38	4	572(369/203)	[26, 27, 29, 34]
miR-320a	48 AAAAGCUGGGUUGAGAGGGCGA 69	3	442 (295/147)	[27, 29, 34]
miR-151a-3p	47 CUAGACUGAAGCUCCUUGAGG 67	3	302 (175/127)	[24, 27, 28]
miR-16	10 UAGCAGCACGUAUUUUUGGCG 31	3	254 (169/85)	[23, 27, 37]
miR-200b-3p	57 UAAUACUGCCUGGUAUGAUGA 78	3	172 (113/59)	[25, 27, 37]
miR-205	34 UCCUUAUCCACCGGAGUCUG 55	3	90 (48/42)	[24, 25, 37]
miR-574	25 UGAGUGUGUGUGUGAGUGUGU 47	3	90 (49/41)	[33, 35, 38]
miR-125b	15 UCCUGAGACCCUAAUUGUGA 36	2	162 (108/54)	[25, 27]
miR-186	15 CAAAGAAUUCUCCUUUUGGCGU 36	2	162 (108/54)	[25, 27]
miR-18a	47 ACGCCCUAAGUGCUCUUCUGG 69	2	156 (91/65)	[26, 38]
Consistently down-regulated miRNA				
miR-1285-3p	51 UCUGGGCAACAAAGUGAGACCU 72	3	280 (143/137)	[24, 25, 28]
miR-1243	5 AACUGGAUCAUUUAUAGGAGUG 26	2	292 (170/122)	[27, 28]
miR-661	51 UGCCUGGGUCUCUGGCCUGCGCGU 74	2	292 (170/122)	[27, 28]
miR-708	11 AAGGAGCUUACAAUCUAGCUGGG 33	2	292 (170/122)	[27, 28]
miR-572	61 GUCCGCUCGGCGGUGGCCCA 80	2	140 (98/42)	[27, 36]
miR-206	53 UGGAAUGUAAGGAAGUGUGUGG 74	2	102 (75/27)	[27, 37]
let-7d	8 AGAGGUAGUAGGUUGCAUAGUU 29	2	84 (45/39)	[36, 38]
miR-15a	14 UAGCAGCACAAUUGGUUUUGUG 35	2	81 (39/42)	[32, 38]
Inconsistently reported miRNA				
miR-20a	8 UAAAGUGCUUAUAGUGCAGGUAG 30	4 (Up)	594 (389/205)	[23, 27, 29, 34]
		2 (Down)	168 (97/71)	[26, 32]
miR-25-3p	52 CAUUGCACUUGUCUCGGUCUGA 73	4 (Up)	497 (312/176)	[27, 29, 34, 38]
		1 (Down)	200 (100/100)	[28]
miR-223	26 CGUGUAUUUGACAAGCUGAGUU 47	4 (Up)	652 (395/257)	[27, 28, 29, 38]
		1 (Down)	48 (28/20)	[36]
let-7f	7 UGAGGUAGUAGAUUGUAUAGUU 28	1 (Up)	200 (100/100)	[28]
		4 (Down)	198 (105/93)	[25, 32, 36, 38]
miR-20b	6 CAAAGUGCUCUAGUGCAGGUAG 28	1 (Up)	92 (70/22)	[27]
		4 (Down)	246 (141/105)	[26, 32, 36, 38]
let-7a	57 CUAUACAAUCUACUGUCUUUC 77	1 (Up)	21 (21/11)	[33]
		3 (Down)	546 (275/ 271)	[25, 31, 38]
miR-17	14 CAAAGUGCUCUACAGUGCAGGUAG 36	1 (Up)	92 (70/22)	[27]
		3 (Down)	650 (316/289)	[26, 31, 32]

<https://doi.org/10.1371/journal.pone.0241593.t002>

miR-145 was significantly down-regulated in NSCLC patients compared with OL patients ($p = 0.003$), HP ($p = 0.038$), and all controls ($p = 0.004$) (Fig 3). miR-20a was significantly up-regulated in NSCLC patients compared with OL patients ($p = 0.045$) and all controls ($p = 0.039$), but not with those in the HP groups. However, no significant difference was observed for other miRNAs.

Serum cytokine concentration in NSCLC patients and controls

Concentrations of tumor-promoting cytokines, including IL-6, VEGF, TGF- β , and TNF- α were determined in the serum of NSCLC patients and controls using ELISA. The results revealed that NSCLC patients had significantly higher VEGF concentrations than those of OL

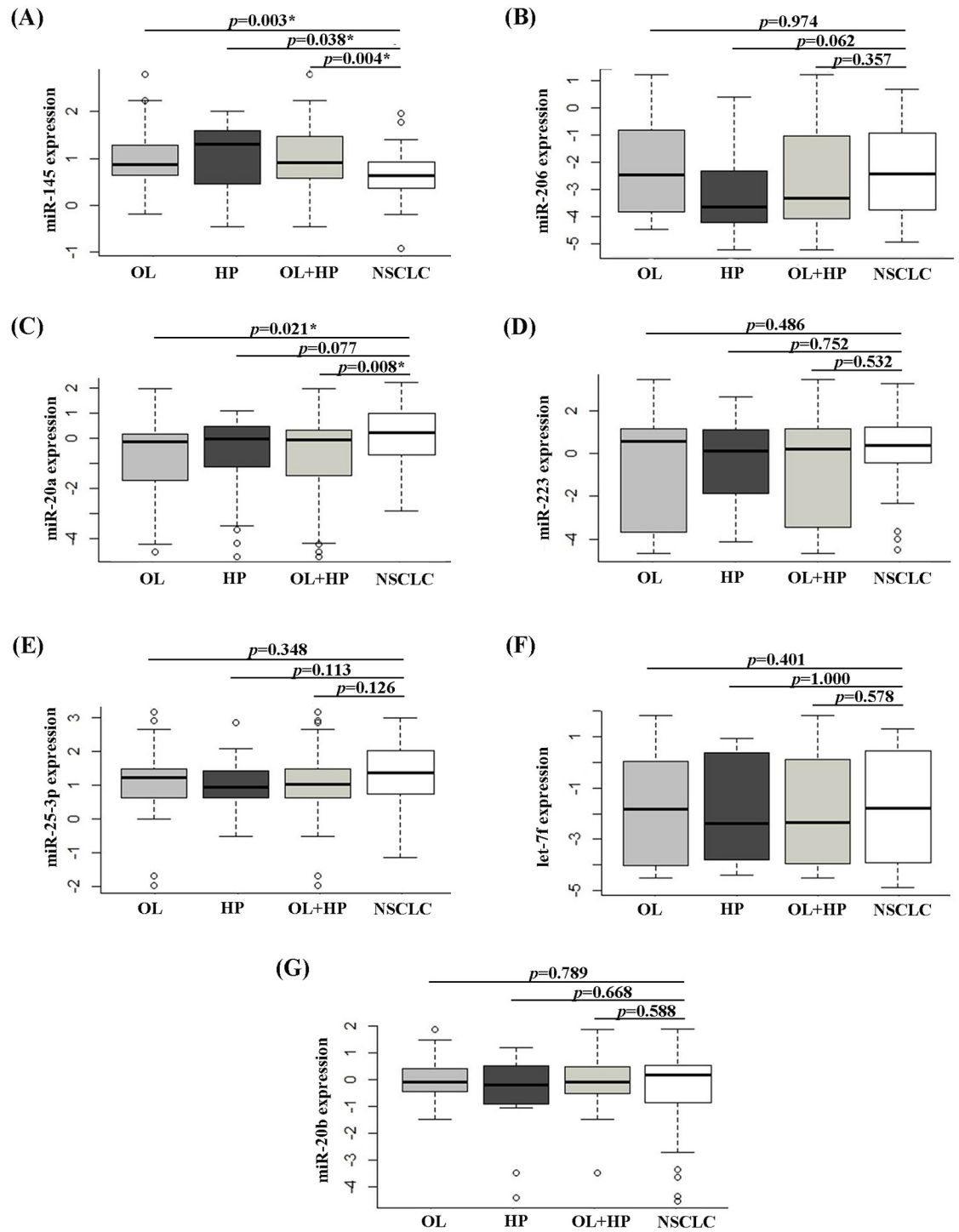


Fig 3. Relative expression of miR-145 (A), miR-206 (B), miR-20a (C), miR-223 (D), miR-25-3p (E), let-7f (F), and miR-20b (G) using qRT-PCR in the serum of NSCLC patients compared with OL patients, HP, and all controls (OL + HP). (*) indicates significant difference of $p < 0.05$.

<https://doi.org/10.1371/journal.pone.0241593.g003>

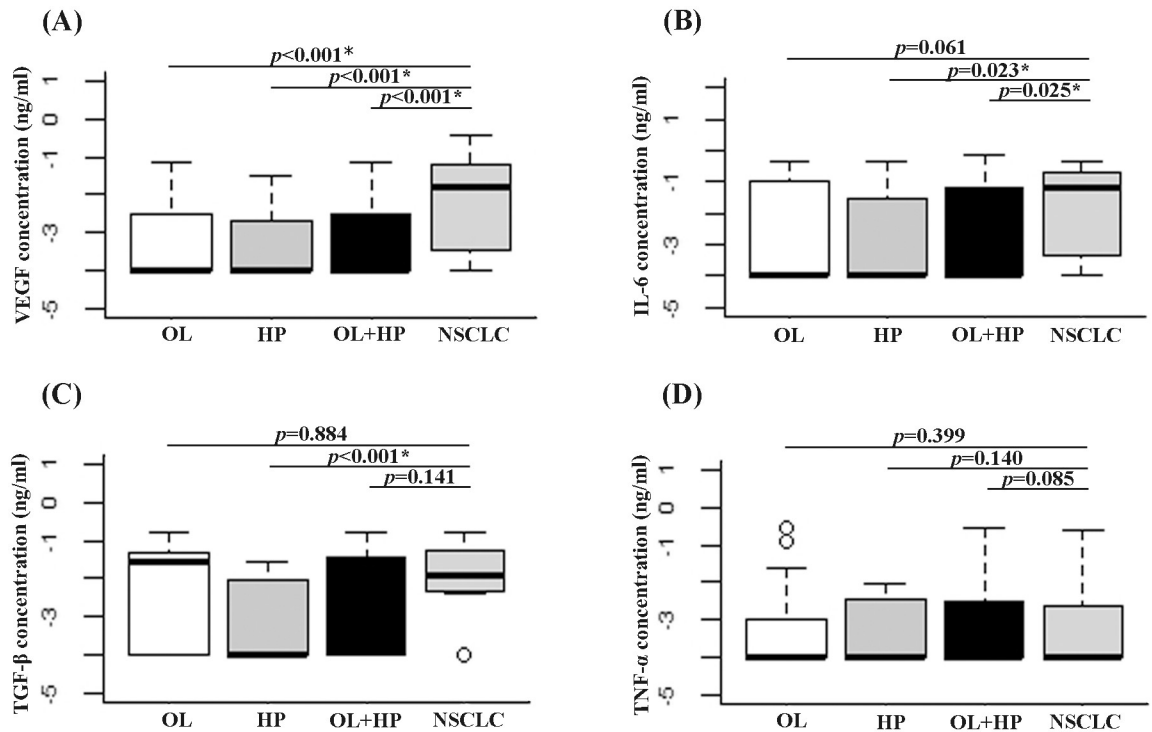


Fig 4. The concentration of VEGF (A), IL-6 (B), TGF- β (C), and TNF- α (D) using ELISA in the serum of NSCLC patients compared with OL patients, HP, and all controls (OL + HP). (*) indicates a significant difference of $p < 0.05$.

<https://doi.org/10.1371/journal.pone.0241593.g004>

and HP ($p < 0.001$) (Fig 4A). A significantly elevated concentration was also found in NSCLC patients compared with the HP group for IL-6 ($p = 0.023$) and TGF- β ($p < 0.001$), and all controls for IL-6 ($p = 0.025$) (Fig 4B and 4C). There was no significant difference in serum TNF- α concentration between NSCLC patients and controls (Fig 4D).

Correlation between miRNA expression and cytokine level

We evaluated the correlation between miRNA expression and cytokine concentration in the serum of NSCLC patients by Spearman's correlation. A moderate correlation was shown between the concentration of TGF- β and the expression level of miR-20a ($r = -0.537$; $p = 0.002$) and miR-223 ($r = 0.428$; $p = 0.015$), in both negative and positive directions, respectively (Fig 5A and 5B). We also detected a weak, positive correlation of VEGF concentration with miR-20a expression ($r = 0.376$; $p = 0.037$) (Fig 5C), whereas miR-223 expression was weakly, negatively correlated with serum VEGF concentration ($r = -0.355$; $p = 0.046$) (Fig 5D). However, the other miRNAs did not exhibit any correlation with cytokines (Table 3).

Discussion

In the present study, the lists of DE miRNAs were identified and then validated in clinical samples. Among 16 lung cancer miRNA profiling studies, eight miRNAs were consistently reported to be differentially expressed in at least three studies. In contrast, seven miRNAs were reported to be differentially expressed with inconsistent direction (either up- or down-regulation) in at least four studies. In the validation study, miR-145 and miR-20a showed a significant difference between NSCLC patients and controls. We also found that the cytokine concentrations of VEGF, IL-6, and TGF- β , but not TNF- α , were higher in NSCLC patients

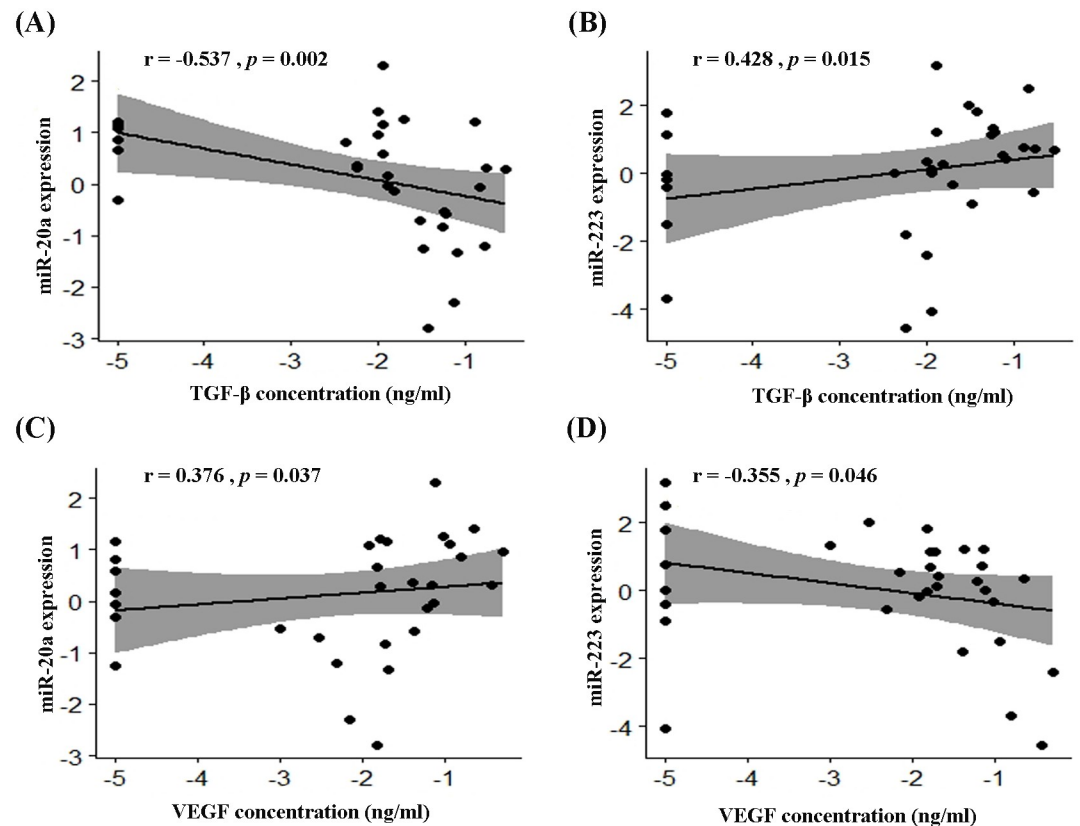


Fig 5. Scatter plots with regression line and 95% CI (gray shade) between miRNA expression and cytokine level in the serum of patients with NSCLC. (A) miR-20a versus TGF- β , (B) miR-223 versus TGF- β , (C) miR-20a versus VEGF, and (D) miR-223 versus VEGF. (*) indicates significant difference of $p < 0.05$ from Spearman's rank correlation.

<https://doi.org/10.1371/journal.pone.0241593.g005>

than in the control groups. In addition, the expression of miR-20a and miR-223 were correlated with the concentrations of TGF- β and VEGF in NSCLC patients.

Although several miRNA profiling studies to identify potential biomarkers for lung cancer have been conducted, the lists of DE miRNAs are not consistent. To deal with this issue, identification based on the vote-counting strategy, which has not yet been reported for lung cancer, was used. We demonstrated that the number of consistent DE miRNAs (79%, 181/229

Table 3. Correlation analysis between miRNA expression and cytokine concentration in the serum of NSCLC patients.

miRNA	IL-6		VEGF		TGF- β		TNF- α	
	r	p-value	r	p-value	r	p-value	r	p-value
miR-145	-0.062	0.738	-0.301	0.107	-0.023	0.899	-0.183	0.467
miR-206	-0.008	0.967	-0.075	0.682	-0.084	0.648	-0.114	0.653
miR-20a	-0.062	0.738	0.376	0.037*	-0.537	0.002*	-0.245	0.327
miR-223	0.037	0.841	-0.355	0.046*	0.428	0.015*	0.268	0.282
miR-25-3p	0.003	0.988	0.237	0.191	-0.272	0.132	-0.110	0.665
let-7f-5p	0.095	0.606	0.147	0.423	-0.022	0.904	-0.186	0.459
miR-20b	-0.156	0.410	0.085	0.645	-0.011	0.951	0.076	0.766

IL-6, interleukin-6; VEGF, vascular endothelial growth factor; TGF- β , transforming growth factor β ; TNF- α , tumor necrosis factor- α .

(*) indicates significant difference of $p < 0.05$.

<https://doi.org/10.1371/journal.pone.0241593.t003>

miRNAs) was higher than those of inconsistent DE miRNAs (21%, 48/229 miRNAs). This result indicated a high degree of concordance among the studies. Inconsistent DE miRNAs could potentially be explained by several factors, such as the diversity of specimens, histological subtypes, clinical stages, and profiling platforms.

From the identification, miR-145 was consistently found to be up-regulated in four studies [26, 27, 29, 34]. However, our validation results in the clinical samples demonstrated the down-regulation of miR-145 in NSCLC patients compared with controls. Previous studies have shown that overexpression of miR-145 increased cell death of prostate cancer cells [39], and inhibited the invasion and metastasis of osteosarcoma cells [40]. Moreover, low expression levels of serum miR-145 predicted poor survival in patients with ovarian cancer [41]. In lung cancer, a decreased expression of miR-145 in tissues was found to have a significantly worse prognosis [42] and associated with a shorter time to relapse in NSCLC [43]. Our finding in clinical validation was consistent with these previous studies, indicating that the miR-145 might play a functional role as tumor-suppressive miRNA.

MiR-20a was found to be up-regulated in four studies [23, 27, 29, 34] and down-regulated in two studies [26, 32]. Our validation study supported the evidence for the up-regulation of miR-20a in NSCLC compared with controls. Although several studies have revealed the aberrant expression of miR-20a in various types of cancer, data on its function is inconsistent. For example, miR-20a overexpression inhibited the invasion of endometrial cancer cells [44] and the proliferation of neuroblastoma cells [45]. In contrast, miR-20a has been found to promote proliferation, invasion, and metastasis in hepatocellular carcinoma [46] and colorectal cancer cells [47]. Regarding clinical studies, increased expression of plasma/serum miR-20a was found in glioblastoma [48], gastric cancer [49], and NSCLC [50]. It was also associated with overall patient survival and disease-free survival. In addition, our bioinformatics analysis revealed that miR-20a regulated several cancer-related genes, particularly tumor suppressor genes, such as PTEN, BCL2L11, FBXO31, and DUSP2. This evidence was in accordance with our result in clinical samples that indicated an oncogenic property of miR-20a in lung cancer.

One important discrepancy between the results in our identification and clinical validation is the stage distribution of NSCLC patients. The studies of Gao *et al.* (2016) [24], Geng *et al.* (2014) [29], and Foss *et al.* (2011) [35] include early-stage NSCLC, while most of the patients in our study were in an advanced stage. This explanation was supported by the study of Aiso *et al.* [51], demonstrating that the serum expressions of miR-145 and miR-20a were higher in the early stages than the advanced stages of NSCLC.

Cytokines have been proposed as blood-based biomarkers and therapeutic targets, because they play essential roles in cancer development, including lung cancer [5]. The increased serum concentration of several cytokines, including VEGF, IL-6, TGF- β , and TNF- α , have been proposed as lung cancer biomarkers [5]. In the present study, we confirmed the increased serum concentration of all cytokines, except TNF- α in NSCLC compared with HP. Since the overestimation of the test could be found if only healthy persons are used as controls [52], both HP and patients with OL patients who may have symptoms similar to lung cancers were also included in this study for comparison.

There are accumulating studies showing that miRNAs associate with tumorigenesis through the regulation of cytokine production [11–13]. We found a moderate correlation between TGF- β concentration and expression levels of miR-20a and miR-223, and a weak correlation between VEGF concentration and expression levels of miR-20a and miR-223. Several studies have demonstrated that miR-20a regulates related genes in TGF- β -signaling, such as TGIF2, E2F5, MYC, ALK5, and TGFBR2 [11, 53]. In addition, some evidence has revealed that miR-20a is associated with VEGF production in breast cancer [12]. For miR-223, Berenstein *et al.* (2016) [10] have shown that decreased expression of miR-223 increases VEGF

expression in bone marrow mesenchymal stromal cells. Liu *et al.* (2018) [54] have observed that miR-223 is increased in TGF- β 1-stimulated cardiac fibroblasts. Our current results support the findings of these previous studies, showing that miR-20a and miR-223 may regulate genes associated with TGF- β and VEGF.

There are some limitations to our present study. First, we included advanced-stage NSCLC for clinical validation. Since the DE miRNAs were identified from miRNA profiling conducted from various stages of NSCLC, the samples from patients with different stages should be more suitable than those from advanced stages of disease in our study. Second, the number of clinical samples, both NSCLC, and controls, is small. This low number may be subject to a type II error (false negative). Therefore, a large clinical sample should be used to assess the diagnostic performance of miR-145 and miR-20a for clinical utility.

Conclusions

We identified miR-145 and miR-20a as potential biomarkers for NSCLC. Our study provides supporting evidence of increased tumor-promoting cytokines in NSCLC, which are correlated with miR-20a and miR-223. Our results suggest that the regulation of tumor-promoting cytokines, through miR-20a and miR-223, might be a new therapeutic approach for lung cancer.

Supporting information

S1 Table. List of the genes associated with the consistently up-regulated miRNA.
(PDF)

S2 Table. List of the genes associated with the consistently down-regulated miRNA.
(PDF)

S3 Table. List of the genes associated with the inconsistency reported miRNA.
(PDF)

Acknowledgments

Research center for cancer control in Thailand are acknowledged for their research facilities. We thank the office of International Affairs, Faculty of Medicine, Prince of Songkla University for English language editing.

Author Contributions

Conceptualization: Sarayut Lucien Geater, Paramee Thongsuksai, Pritsana Raungrut.

Data curation: Keson Trakunran, Sarayut Lucien Geater, Warangkana Keeratichananont, Pritsana Raungrut.

Formal analysis: Pichitpon Chaniad, Keson Trakunran, Paramee Thongsuksai, Pritsana Raungrut.

Investigation: Pichitpon Chaniad, Pritsana Raungrut.

Supervision: Pritsana Raungrut.

Validation: Pichitpon Chaniad.

Writing – original draft: Pichitpon Chaniad, Pritsana Raungrut.

Writing – review & editing: Paramee Thongsuksai, Pritsana Raungrut.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018; 68: 394–424. <https://doi.org/10.3322/caac.21492> PMID: 30207593
2. Howlader N, Noone AM, Krapcho M, Miller D, Brest A, Yu M, et al. SEER Cancer Statistics Review, 1975–2016 Bethesda, MD: National Cancer Institute; April 2019 [https://seer.cancer.gov/csr/1975_2016/].
3. Nakamura K, Ukawa S, Okada E, Hirata M, Nagai A, Yamagata Z, et al. Characteristics and prognosis of Japanese male and female lung cancer patients: The BioBank Japan Project. *J Epidemiol*. 2017; 27: S49–s57. <https://doi.org/10.1016/j.je.2016.12.010> PMID: 28202209
4. Song XY, Zhou SJ, Xiao N, Li YS, Zhen DZ, Su CY, et al. Research on the relationship between serum levels of inflammatory cytokines and non-small cell lung cancer. *Asian Pac J Cancer Prev*. 2013; 14: 4765–4768. <https://doi.org/10.7314/apjcp.2013.14.8.4765> PMID: 24083740
5. Marrugal A, Ojeda L, Paz-Ares L, Molina-Pinelo S, Ferrer I. Proteomic-Based Approaches for the Study of Cytokines in Lung Cancer. *Dis Markers*. 2016; 2016: 2138627. <https://doi.org/10.1155/2016/2138627> PMID: 27445423
6. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004; 116: 281–297. [https://doi.org/10.1016/s0092-8674\(04\)00045-5](https://doi.org/10.1016/s0092-8674(04)00045-5) PMID: 14744438
7. Fan J, Kou X, Yang Y, Chen N. MicroRNA-Regulated Proinflammatory Cytokines in Sarcopenia. *Mediators Inflamm*. 2016; 2016: 1438686. <https://doi.org/10.1155/2016/1438686> PMID: 27382188
8. Trakunram K, Champoochana N, Chaniad P, Thongsuksai P, Raungrut P. MicroRNA Isolation by Trizol-Based Method and Its Stability in Stored Serum and cDNA Derivatives. *Asian Pac J Cancer Prev*. 2019; 20: 1641–1647. <https://doi.org/10.31557/APJCP.2019.20.6.1641> PMID: 31244282
9. Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, et al. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF- α stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol*. 2007; 179: 5082–5089. <https://doi.org/10.4049/jimmunol.179.8.5082> PMID: 17911593
10. Berenstein R, Nogai A, Waechter M, Blau O, Kuehnel A, Schmidt-Hieber M, et al. Multiple myeloma cells modify VEGF/IL-6 levels and osteogenic potential of bone marrow stromal cells via Notch/miR-223. *Mol Carcinog*. 2016; 55: 1927–1939. <https://doi.org/10.1002/mc.22440> PMID: 27023728
11. Pellatt AJ, Mullany LE, Herrick JS, Sakoda LC, Wolff RK, Samowitz WS, et al. The TGF β -signaling pathway and colorectal cancer: associations between dysregulated genes and miRNAs. *J Transl Med*. 2018; 16: 191. <https://doi.org/10.1186/s12967-018-1566-8> PMID: 29986714
12. Luengo-Gil G, Gonzalez-Billalabeitia E, Perez-Henarejos SA, Navarro Manzano E, Chaves-Benito A, Garcia-Martinez E, et al. Angiogenic role of miR-20a in breast cancer. *PLoS One*. 2018; 13: e0194638. <https://doi.org/10.1371/journal.pone.0194638> PMID: 29617404
13. Yang Y, Ding L, Hu Q, Xia J, Sun J, Wang X, et al. MicroRNA-218 functions as a tumor suppressor in lung cancer by targeting IL-6/STAT3 and negatively correlates with poor prognosis. *Mol Cancer*. 2017; 16: 141. <https://doi.org/10.1186/s12943-017-0710-z> PMID: 28830450
14. Chan SK, Griffith OL, Tai IT, Jones SJ. Meta-analysis of colorectal cancer gene expression profiling studies identifies consistently reported candidate biomarkers. *Cancer Epidemiol Biomarkers Prev*. 2008; 17: 543–552. <https://doi.org/10.1158/1055-9965.EPI-07-2615> PMID: 18349271
15. Griffith OL, Melck A, Jones SJ, Wiseman SM. Meta-analysis and meta-review of thyroid cancer gene expression profiling studies identifies important diagnostic biomarkers. *J Clin Oncol*. 2006; 24: 5043–5051. <https://doi.org/10.1200/JCO.2006.06.7330> PMID: 17075124
16. Chou CH, Shrestha S, Yang CD, Chang NW, Lin YL, Liao KW, et al. miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions. *Nucleic Acids Res*. 2018; 46: D296–d302. <https://doi.org/10.1093/nar/gkx1067> PMID: 29126174
17. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I, et al. DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic Acids Res*. 2015; 43: D153–159. <https://doi.org/10.1093/nar/gku1215> PMID: 25416803
18. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003; 13: 2498–2504. <https://doi.org/10.1101/gr.1239303> PMID: 14597658
19. Krupke DM, Begley DA, Sundberg JP, Richardson JE, Neuhauser SB, Bult CJ. The Mouse Tumor Biology Database: A Comprehensive Resource for Mouse Models of Human Cancer. *Cancer Res*. 2017; 77: e67–e70. <https://doi.org/10.1158/0008-5472.CAN-17-0584> PMID: 29092943
20. Travis WD, Brambilla E, Nicholson AG, Yatabe Y, Austin JHM, Beasley MB, et al. The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic

- Advances Since the 2004 Classification. *J Thorac Oncol*. 2015; 10: 1243–1260. <https://doi.org/10.1097/JTO.0000000000000630> PMID: 26291008
21. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol*. 2010; 17: 1471–1474. <https://doi.org/10.1245/s10434-010-0985-4> PMID: 20180029
 22. Rao X, Huang X, Zhou Z, Lin X. An improvement of the 2^{−ΔΔCT} method for quantitative real-time polymerase chain reaction data analysis. *Biostat Bioinforma Biomath*. 2013; 3: 71–85. PMID: 25558171
 23. Fan L, Qi H, Teng J, Su B, Chen H, Wang C, et al. Identification of serum miRNAs by nano-quantum dots microarray as diagnostic biomarkers for early detection of non-small cell lung cancer. *Tumour Biol*. 2016; 37: 7777–7784. <https://doi.org/10.1007/s13277-015-4608-3> PMID: 26695145
 24. Gao X, Wang Y, Zhao H, Wei F, Zhang X, Su Y, et al. Plasma miR-324-3p and miR-1285 as diagnostic and prognostic biomarkers for early stage lung squamous cell carcinoma. *Oncotarget*. 2016; 7: 59664–59675. <https://doi.org/10.18632/oncotarget.11198> PMID: 27517633
 25. Halvorsen AR, Bjaanaes M, LeBlanc M, Holm AM, Bolstad N, Rubio L, et al. A unique set of 6 circulating microRNAs for early detection of non-small cell lung cancer. *Oncotarget*. 2016; 7: 37250–37259. <https://doi.org/10.18632/oncotarget.9363> PMID: 27191990
 26. Leidinger P, Brefort T, Backes C, Krapp M, Galata V, Beier M, et al. High-throughput qRT-PCR validation of blood microRNAs in non-small cell lung cancer. *Oncotarget*. 2016; 7: 4611–4623. <https://doi.org/10.18632/oncotarget.6566> PMID: 26672767
 27. Nadal E, Truini A, Nakata A, Lin J, Reddy RM, Chang AC, et al. A Novel Serum 4-microRNA Signature for Lung Cancer Detection. *Sci Rep*. 2015; 5: 12464. <https://doi.org/10.1038/srep12464> PMID: 26202143
 28. Wozniak MB, Scelo G, Muller DC, Mukeria A, Zaridze D, Brennan P. Circulating MicroRNAs as Non-Invasive Biomarkers for Early Detection of Non-Small-Cell Lung Cancer. *PLoS One*. 2015; 10: e0125026. <https://doi.org/10.1371/journal.pone.0125026> PMID: 25965386
 29. Geng Q, Fan T, Zhang B, Wang W, Xu Y, Hu H. Five microRNAs in plasma as novel biomarkers for screening of early-stage non-small cell lung cancer. *Respir Res*. 2014; 15: 149. <https://doi.org/10.1186/s12931-014-0149-3> PMID: 25421010
 30. Rani S, Gately K, Crown J, O'Byrne K, O'Driscoll L. Global analysis of serum microRNAs as potential biomarkers for lung adenocarcinoma. *Cancer Biol Ther*. 2013; 14: 1104–1112. <https://doi.org/10.4161/cbt.26370> PMID: 24025412
 31. Heegaard NH, Schetter AJ, Welsh JA, Yoneda M, Bowman ED, Harris CC. Circulating micro-RNA expression profiles in early stage nonsmall cell lung cancer. *Int J Cancer*. 2012; 130: 1378–1386. <https://doi.org/10.1002/ijc.26153> PMID: 21544802
 32. Patnaik SK, Yendamuri S, Kannisto E, Kucharczuk JC, Singhal S, Vachani A. MicroRNA expression profiles of whole blood in lung adenocarcinoma. *PLoS One*. 2012; 7: e46045. <https://doi.org/10.1371/journal.pone.0046045> PMID: 23029380
 33. Roth C, Stuckrath I, Pantel K, Izbicki JR, Tachezy M, Schwarzenbach H. Low levels of cell-free circulating miR-361-3p and miR-625* as blood-based markers for discriminating malignant from benign lung tumors. *PLoS One*. 2012; 7: e38248. <https://doi.org/10.1371/journal.pone.0038248> PMID: 22675530
 34. Chen X, Hu Z, Wang W, Ba Y, Ma L, Zhang C, et al. Identification of ten serum microRNAs from a genome-wide serum microRNA expression profile as novel noninvasive biomarkers for nonsmall cell lung cancer diagnosis. *Int J Cancer*. 2012; 130: 1620–1628. <https://doi.org/10.1002/ijc.26177> PMID: 21557218
 35. Foss KM, Sima C, Ugolini D, Neri M, Allen KE, Weiss GJ. miR-1254 and miR-574-5p: serum-based microRNA biomarkers for early-stage non-small cell lung cancer. *J Thorac Oncol*. 2011; 6: 482–488. <https://doi.org/10.1097/JTO.0b013e318208c785> PMID: 21258252
 36. Silva J, Garcia V, Zaballos A, Provencio M, Lombardia L, Almonacid L, et al. Vesicle-related microRNAs in plasma of nonsmall cell lung cancer patients and correlation with survival. *Eur Respir J*. 2011; 37: 617–623. <https://doi.org/10.1183/09031936.00029610> PMID: 20595154
 37. Wang ZX, Bian HB, Wang JR, Cheng ZX, Wang KM, De W. Prognostic significance of serum miRNA-21 expression in human non-small cell lung cancer. *J Surg Oncol*. 2011; 104: 847–851. <https://doi.org/10.1002/jso.22008> PMID: 21721011
 38. Keller A, Leidinger P, Borries A, Wendschlag A, Wucherpfennig F, Scheffler M, et al. miRNAs in lung cancer—Studying complex fingerprints in patient's blood cells by microarray experiments. *BMC Cancer*. 2009; 9: 353. <https://doi.org/10.1186/1471-2407-9-353> PMID: 19807914
 39. Chen X, Gong J, Zeng H, Chen N, Huang R, Huang Y, et al. MicroRNA145 targets BNIP3 and suppresses prostate cancer progression. *Cancer Res*. 2010; 70: 2728–2738. <https://doi.org/10.1158/0008-5472.CAN-09-3718> PMID: 20332243

40. Fan L, Wu Q, Xing X, Wei Y, Shao Z. MicroRNA-145 targets vascular endothelial growth factor and inhibits invasion and metastasis of osteosarcoma cells. *Acta Biochim Biophys Sin (Shanghai)*. 2012; 44: 407–414. <https://doi.org/10.1093/abbs/gms019> PMID: 22472569
41. Liang H, Jiang Z, Xie G, Lu Y. Serum microRNA-145 as a novel biomarker in human ovarian cancer. *Tumour Biol*. 2015; 36: 5305–5313. <https://doi.org/10.1007/s13277-015-3191-y> PMID: 25722112
42. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell*. 2006; 9: 189–198. <https://doi.org/10.1016/j.ccr.2006.01.025> PMID: 16530703
43. Campayo M, Navarro A, Vinolas N, Diaz T, Tejero R, Gimferrer JM, et al. Low miR-145 and high miR-367 are associated with unfavourable prognosis in resected nonsmall cell lung cancer. *Eur Respir J*. 2013; 41: 1172–1178. <https://doi.org/10.1183/09031936.00048712> PMID: 22835608
44. Huang Y, Yang N. MicroRNA-20a-5p inhibits epithelial to mesenchymal transition and invasion of endometrial cancer cells by targeting STAT3. *Int J Clin Exp Pathol* 2018; 11: 5715–5724. PMID: 31949657
45. Yu Y, Zhang J, Jin Y, Yang Y, Shi J, Chen F, et al. MiR-20a-5p suppresses tumor proliferation by targeting autophagy-related gene 7 in neuroblastoma. *Cancer Cell Int*. 2018; 18: 5. <https://doi.org/10.1186/s12935-017-0499-2> PMID: 29311760
46. Chen Y, Wang X, Cheng J, Wang Z, Jiang T, Hou N, et al. MicroRNA-20a-5p targets RUNX3 to regulate proliferation and migration of human hepatocellular cancer cells. *Oncol Rep*. 2016; 36: 3379–3386. <https://doi.org/10.3892/or.2016.5144> PMID: 27748919
47. Cheng D, Zhao S, Tang H, Zhang D, Sun H, Yu F, et al. MicroRNA-20a-5p promotes colorectal cancer invasion and metastasis by downregulating Smad4. *Oncotarget*. 2016; 7: 45199–45213. <https://doi.org/10.18632/oncotarget.9900> PMID: 27286257
48. Zhao H, Shen J, Hodges TR, Song R, Fuller GN, Heimberger AB. Serum microRNA profiling in patients with glioblastoma: a survival analysis. *Mol Cancer*. 2017; 16: 59. <https://doi.org/10.1186/s12943-017-0628-5> PMID: 28284220
49. Yang R, Fu Y, Zeng Y, Xiang M, Yin Y, Li L, et al. Serum miR-20a is a promising biomarker for gastric cancer. *Biomed Rep*. 2017; 6: 429–434. <https://doi.org/10.3892/br.2017.862> PMID: 28413641
50. Xu X, Zhu S, Tao Z, Ye S. High circulating miR-18a, miR-20a, and miR-92a expression correlates with poor prognosis in patients with non-small cell lung cancer. *Cancer Med*. 2018; 7: 21–31. <https://doi.org/10.1002/cam4.1238> PMID: 29266846
51. Aiso T, Ohtsuka K, Ueda M, Karita S, Yokoyama T, Takata S, et al. Serum levels of candidate microRNA diagnostic markers differ among the stages of non-small-cell lung cancer. *Oncol Lett*. 2018; 16: 6643–6651. <https://doi.org/10.3892/ol.2018.9464> PMID: 30405804
52. Rutjes AW, Reitsma JB, Vandenbroucke JP, Glas AS, Bossuyt PM. Case-control and two-gate designs in diagnostic accuracy studies. *Clin Chem*. 2005; 51: 1335–1341. <https://doi.org/10.1373/clinchem.2005.048595> PMID: 15961549
53. Correia AC, Moonen JR, Brinker MG, Krenning G. FGF2 inhibits endothelial-mesenchymal transition through microRNA-20a-mediated repression of canonical TGF-beta signaling. *J Cell Sci*. 2016; 129: 569–579. <https://doi.org/10.1242/jcs.176248> PMID: 26729221
54. Liu X, Xu Y, Deng Y, Li H. MicroRNA-223 Regulates Cardiac Fibrosis After Myocardial Infarction by Targeting RASA1. *Cell Physiol Biochem*. 2018; 46: 1439–1454. <https://doi.org/10.1159/000489185> PMID: 29689569