Pathology - Research and Practice 215 (2019) 152466

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Detection of circulating exosomal miR-17-5p serves as a novel non-invasive diagnostic marker for non-small cell lung cancer patients

Yi Zhang[a], Yingmei Zhang[b], Yunhong Yin[a], Shuhai Li[c,*]

[a] Department of Respiratory and Critical Care Medicine, Qilu Hospital of Shandong University, wen hua xi lu 107#, Jinan, 250012, Shandong Province, China
[b] Department of Respiratory Medicine, Linyi People's Hospital, jie fang lu dong duan 27#, Linyi, 276000, Shandong Province, China
[c] Department of Thoracic Surgery, Qilu Hospital of Shandong University, wen hua xi lu 107#, Jinan, 250012, Shandong Province, China

ARTICLE INFO

Keywords:
NSCLC
Serum
Exosome
miRNA
Diagnosis

ABSTRACT

Exosome-shuttled bioactive miRNAs act as novel non-invasive biomarkers for cancer diagnosis have received increasing attention. In this study, we aimed to investigate the expression signatures of exosomal miRNAs and develop a serum exosome-derived miRNA panel for diagnosis of non-small cell lung cancer (NSCLC). The miR-17-92 cluster including 6 miRNAs (miR-17-5p, miR-18a-5p, miR-19a-3p, miR-19b-1-5p, miR-20a-5p and miR-92a-1-5p) was selected as potential diagnostic candidate molecule. Then, expression profiles of the candidate miRNAs were firstly analyzed in 43 pairs of serum samples from the training set by quantitative real-time PCR, and the dysregulated miRNA along with three tumor markers (carcinoembryonic antigen, CEA; cytokeratin 19 fragment, CYFRA21-1; squamous cell carcinoma antigen, SCCA) were further validated in two independent cohorts, which consisted of training set (including 100 NSCLC patients and 90 healthy controls) and validation set (including 72 NSCLC patients and 47 healthy controls). The expression of miR-17-5p was significantly up-regulated in NSCLC patients compared with the healthy controls ($P < 0.001$), suggesting that miR-17-5p might have considerable clinical value in the diagnosis of NSCLC. Based on the data from the training set, we next used a logistic regression model to construct a 4-molecule panel consisting of miR-17-5p and three tumor markers for NSCLC diagnosis. The performance of such 4-molecule panel was verified with an area under the ROC curve of 0.860 (95% CI = 0.802 to 0.906, sensitivity = 63.0% and specificity = 93.3%) and 0.844 (95% CI = 0.766 to 0.904, sensitivity = 76.4% and specificity = 76.6%) in the training set and validation set, respectively. In conclusion, the newly developed diagnostic panel consisting of exosomal miR-17-5p, CEA, CYFRA21-1 and SCCA may have considerable clinical value in the diagnosis of NSCLC.

1. Introduction

Lung cancer still remains the leading cause of cancer-related death all over the world, of which, non-small cell lung cancer (NSCLC) accounts for more than 80% [1,2]. Despite continual progress has been made in the multidisciplinary therapy, the 5-year survival rate is still relatively poor; early diagnosis could significantly improve the prognosis, however, the disease has progressed to advanced stage in most patients when firstly diagnosed [3,4]. Hence, it is clinically urgent to make an early screening and definitive diagnosis for NSCLC. The imageological screening generally is expensive and with low sensitivity in identifying cancer cells at an early stage [5,6]. In addition, biopsy for histopathologic examination is not suit for screening due to invasiveness. By contrast, peripheral blood test owns superior advantages in the field of cancer screening for the characters of non-invasive, accessible, high-efficiency and so forth. Meanwhile, the sensitivity or specificity of conventional-used markers for NSCLC is still disappointing [7]. Altogether, original and effective biomarkers which can improve the early diagnosis of NSCLC are eagerly required.

MicroRNAs (miRNAs) are an abundant class of endogenous non-coding small RNA, act as tumor oncogene or repressor in clusters by facilitating degradation or translational repression of the target mRNAs [8,9]. Growing evidences have demonstrated that miRNA clusters caper a momentous role in carcinogenesis. For instance, miR-17-92 cluster including 6 different miRNAs encoded by a polycistron RNA expressed aberrantly and involved in the pathological process of various cancers

Abbreviations: NSCLC, non-small cell lung cancer; miRNAs, microRNAs; CEA, carcinoembryonic antigen; CYFRA21-1, cytokeratin 19 fragment; SCCA, squamous cell carcinoma antigen; TEM, transmission electron microscopy; PBS, phosphate buffered solution; NTA, nanoparticle tracking analysis; TSG101, tumor susceptibility gene 101; HRP, horseradish peroxidase; SE, serum exosome

* Corresponding author.
E-mail address: lishuhai@sdu.edu.cn (S. Li).

https://doi.org/10.1016/j.prp.2019.152466
Received 1 March 2019; Received in revised form 12 May 2019; Accepted 21 May 2019

[10–12]. Recently, miR-18a-5p, one member of miR-17-92 cluster, was reported to act as an oncogene by directly suppressing interferon regulatory factor 2 in lung cancer [13]. It has to be underlined that miR-17-92 cluster could manoeuvre multiple molecular mechanisms to regulate its target genes, and there is no related reports regarding the biological roles of miR-17-92 cluster in NSCLC so far.

Exosomes are lipid vesicles derived from endosome, cancer cells secrete exosomes to exchange significant information and then promote tumor progression [14]. It has been confirmed that miRNA could be parceled into exosomes and circulate in stable cell-free form in multifarious body fluids [15,16]. There has been ever-increasing interest in investigating the usefulness of exosome-encapsulated miRNAs which may serve as potential diagnostic and prognostic biomarkers, or therapeutic targets in cancers [17–20]. Recently, exosome miRNAs have received more attention as potent biomarkers for differential diagnosis of lung cancer [21,22]. However, to the best of our knowledge, expression of the circulating exosome miR-17-92 cluster in correlation with diagnostic biomarker for NSCLC has not been elucidated so far.

In this study, we detected the expression profile of miR-17-92 cluster in exosomes derived from the serum of NSCLC patients, further to build a diagnostic panel combined with carcinoembryonic antigen (CEA), cytokeratin 19 fragment (CYFRA21-1) and squamous cell carcinoma antigen (SCCA) in order to achieve the optimal diagnosis of NSCLC with high sensitivity or specificity for the first time.

2. Materials and methods

2.1. Patients and healthy controls

A total of 172 NSCLC patients (131 cases of adenocarcinoma and 41 cases of squamous cell carcinoma) did not receive chemotherapy or radiotherapy before serum collection were enrolled in this study from the Qilu Hospital of Shandong University between January 2016 and October 2018. Samples of healthy controls were acquired from people for health examination from the same hospital. Clinical features of NSCLC patients and healthy controls were listed in Table 1. The pathological stage classification of NSCLC was determined based on the eighth edition of the TNM staging system [23]. A written informed consent was obtained from each participant for experimentation with human subjects, and this study was approved by the institutional ethics committee of Qilu Hospital of Shandong University.

Serum isolated from whole blood was immediately transferred to a 1.5 ml eppendorf tube and subjected to a two-step centrifugation (1500 *g* for 10 min at 4℃ and then 13,800 *g* for 15 min at 4℃) to eliminate cell sediments. One aliquot of the serum sample was used within 2 h to detect the expressions of tumor markers (CEA, CYFRA21-1 and SCCA), while a second aliquot was stored at -80℃ in an RNase-free tube until total RNA extraction.

2.2. Exosomes and RNA extraction

Exosomes were extracted from the serum samples using the exosome precipitation solution (ExoQuick™, SBI, USA) according to the manufacturer's instructions. Then total exosomal RNA was extracted from the above exosomes using the miRNeasy Micro Kit (QIAGEN, Product# 217084, Germany) according to the manufacturer's instructions and evaluated by a NanoDrop spectrophotometer (Thermo Fisher Scientific).

2.3. qRT-PCR

Purified RNA (< 1000 ng) was reversely transcribed into cDNA using the All-in-one™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, USA) on a SimpliAmp™ Thermal Cycler (ABI, Singapore) according to the manufacturer's instructions. The reaction mixture was incubated at 37℃ for 60 min, followed by 85℃ for 5 s and 4℃ for 60 min. qRT-PCR was performed in a 25-μL reaction system using the All-in-one™ miRNA qPCR Kit (GeneCopoeia, USA). This reaction was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, after an initial denaturation step at 95℃ for 10 min, the amplifications were carried out with 40 cycles at a melting temperature of 95℃ for 10 s and an annealing temperature of 60℃ for 20 s. The specificity of the PCR products was evaluated through melting curve analysis. MiR-16-5p was selected as the reference gene, and all primers sequences involved in this study were provided in the Table S1. The relative expressions of target gene were calculated by comparative cycle threshold (Ct) ($2^{-\Delta\Delta Ct}$) method.

Table 1
Clinicopathological characteristics of patients and demographic information of controls in training set and validation set.

Variable	Training set n = 190	Validation set n = 119	*P* value
Control (number)	90	47	
Age (years)[a]			0.48
≤53	44	26	
> 53	46	21	
Sex			0.35
Male	44	19	
Female	46	28	
NSCLC (number)	100	72	
Age (years)[b]			0.09
≤61	59	33	
> 61	41	39	
Sex			0.21
Male	50	43	
Female	50	29	
Tumor size (diameter)			0.42
≤3cm	41	34	
> 3cm	59	38	
Lymph node metastasis			0.06
Negative	54	49	
Positive	46	23	
TNM stage			0.06
I	41	29	
II	23	27	
III	36	16	

NSCLC: non-small cell lung cancer.
[a] Median of control participants in training set.
[b] Median of patients in training set.

2.4. Transmission electron microscopy (TEM)

Exosomes extracted from serum samples were first resuspended in 200 μl phosphate buffered solution (PBS), then 20ul exosomal suspension on the ice and were placed on a glow discharged 200-mesh Cu grid coated with carbon-Formvar film (ProSciTech, Kirwan, QLD, Australia) and allowed to absorb for 1 min (clamping the Cu grid with tweezers should be light to prevent the copper network from being broken). Subsequently, the sample was dried with filter paper and stained with 20ul 2% uranyl acetate aqueous solution at room temperature for 1 min. Finally, dried the dye solution along the edge with filter paper and baked the Cu grid under an infrared lamp for 10 min. Sample was imaged using a Tecnai G2 Spirit (FEI) TEM at 300 kV across 15,000 to 36,000 magnifications.

2.5. Nanoparticle tracking analysis (NTA)

NTA was selected to detect the size distribution and concentration of exosomes. Briefly, exosomes were diluted in 1 ml PBS and mixed well, and then a certain volume of exosomal suspension was injected into the ZETASIZER Nano series-Nano-ZS instrument (Malvern, UK). Particles were automatically tracked and sized based on the Brownian motion and diffusion coefficient. Filtered PBS was used as a control.

2.6. Western blotting analysis

Total protein of serum exosomes was extracted with RIPA (Solarbio, China, #R0010) according to the manufacturer's instructions. Equal amounts of protein lysates were subjected to western blotting analysis by using anti-CD9 antibody (rabbit IgG, 13174S, CST, USA), anti-tumor susceptibility gene 101 (TSG101) (mouse IgG, Ab83, Abcam, UK), goat anti-rabbit horseradish peroxidase (HRP) secondary antibody (ZB-2301, ZSGB-BIO, China) and goat anti-mouse HRP secondary antibody (ZB-2305, ZSGB-BIO, China) according to standard protocols as previously described. Immunoreactive bands were visualized using the Clarity Western ECL kit (Bio-Rad).

2.7. Statistical analysis

SPSS Statistics 22.0 (IBM, Chicago, IL, USA) was used to assess all miRNA expression data to ascertain normal distribution. Nonparametric Mann–Whitney U test was used to compare the differences in miRNA expression between the NSCLC and healthy controls. GraphPad Prism 5 (San Diego, CA, USA) was selected to generate scatter diagrams. Logistic regression analysis was performed utilizing MATLAB software (MATLAB, R2014a, Natick, MA, USA) to establish the diagnostic panel. ROC curves were generated using Medcalc 15.2.2 (Med-Calc, Mariakerke, Belgium), and the area under the curve (AUC) was utilized to evaluate the feasibility of miRNA to differentially diagnose between NSCLC and healthy controls. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of serum exosomes

TEM was used to directly observe the morphology of exosomes, and exosomes were found to have a diameter of 60–150 nm and a cup-shaped membrane (Fig. 1a). Nano Sight nanoparticle characterization system revealed that the average particle size of serum exosomes and the range of the main peak were in the scope of exosomes. Particles ranging from 20 nm to 200 nm in diameter accounted for 98.1%, which was consistent with the exosomal particle size distribution (Fig. 1b). In addition, in order to further identify serum exosomes, western blotting analysis was performed to detect the established markers on the surface of exosomes, including CD9 and TSG101 (Fig. 1c).

Table 2
Expression of candidate serum exosome-derived miRNAs in NSCLC patients and healthy controls [median (interquartile range)].

miRNA	Training set		
	Controls	NSCLCs	P Value
miR-92a-1-5p	0.970 (0.435-2.470)	2.010 (1.048-3.610)	0.064
miR-17-5p	1.288 (0.630-1.972)	3.117 (0.983-10.320)	< 0.001
miR-18a-5p	1.185 (0.465-1.895)	1.150 (0.930-0.930)	0.573
miR-19a-3p	1.555 (0.160-6.343)	3.610 (0.330-7.780)	0.220
miR-19b-1-5p	1.712 (0.153-4.352)	3.283 (0.975-5.860)	0.090
miR-20a-5p	0.987 (0.579-1.628)	1.339 (0.897-2.021)	0.119

NSCLC: non-small cell lung cancer.

3.2. Screening and evaluation of serum exosome (SE)-derived dysregulated miRNAs in NSCLC patients

The miR-17-92 cluster including 6 miRNAs (miR-17-5p, miR-18a-5p, miR-19a-3p, miR-19b-1-5p, miR-20a-5p and miR-92a-1-5p) was selected as potential diagnostic biomarkers. qRT-PCR was performed in two independent cohorts, which consisted of training set (including 100 NSCLC patients and 90 healthy controls) and validation set (including 72 NSCLC patients and 47 healthy controls), to analyze the expressions of these 6 candidate miRNAs in serum exosomes. In the training set, our data showed that miR-17-5p was significantly up-regulated in NSCLC patients compared with the healthy controls ($P < 0.001$, Table 2, Fig. 2a). The rest of the 5 candidate miRNAs were not significantly different between the two groups (including 43 NSCLC patients and 43

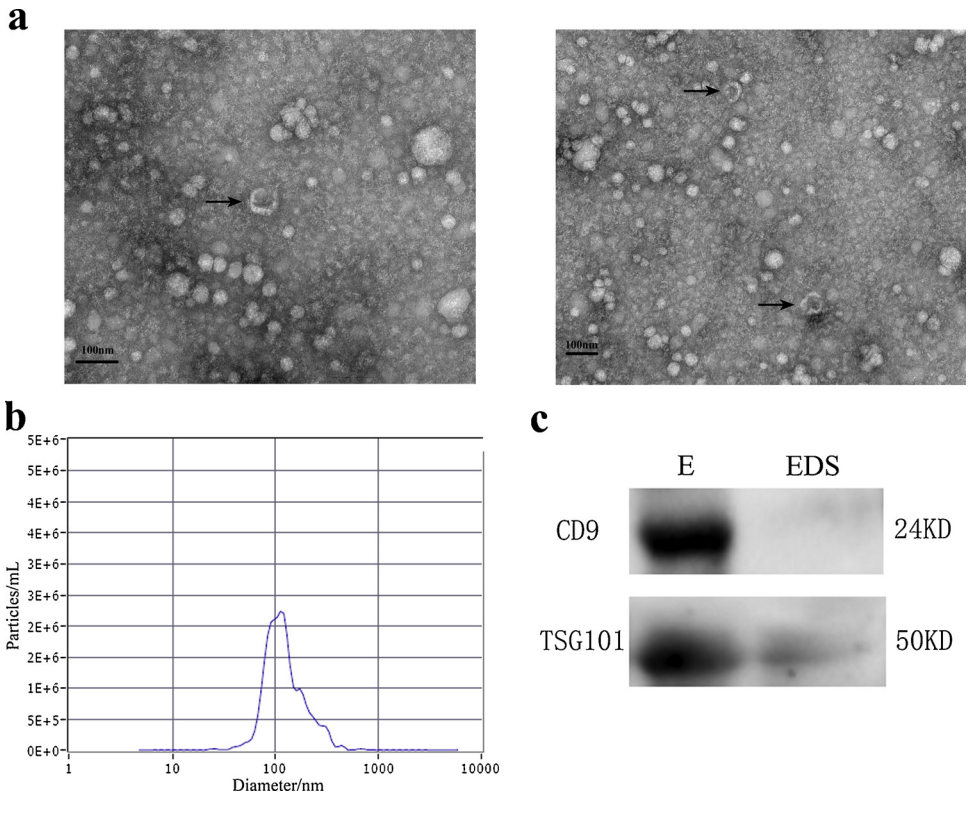

Fig. 1. Characterization of SEs. (a) TEM was used to analyze SEs, which exhibited a cup-shaped membrane morphology with a diameter of 60–150 nm. Typical exosomes were shown with the black arrows. (b) The NTA characterization system was used to characterize the sizes of serum exosomes, and results showed that the majority of vesicle particles were mainly between 60 and 150 nm in diameter. (c) Western blotting was performed to analyze the exosomal protein markers including CD9 (24KD) and TSG101 (50KD) in exosomes (E) and exosome-depleted supernatant (EDS).

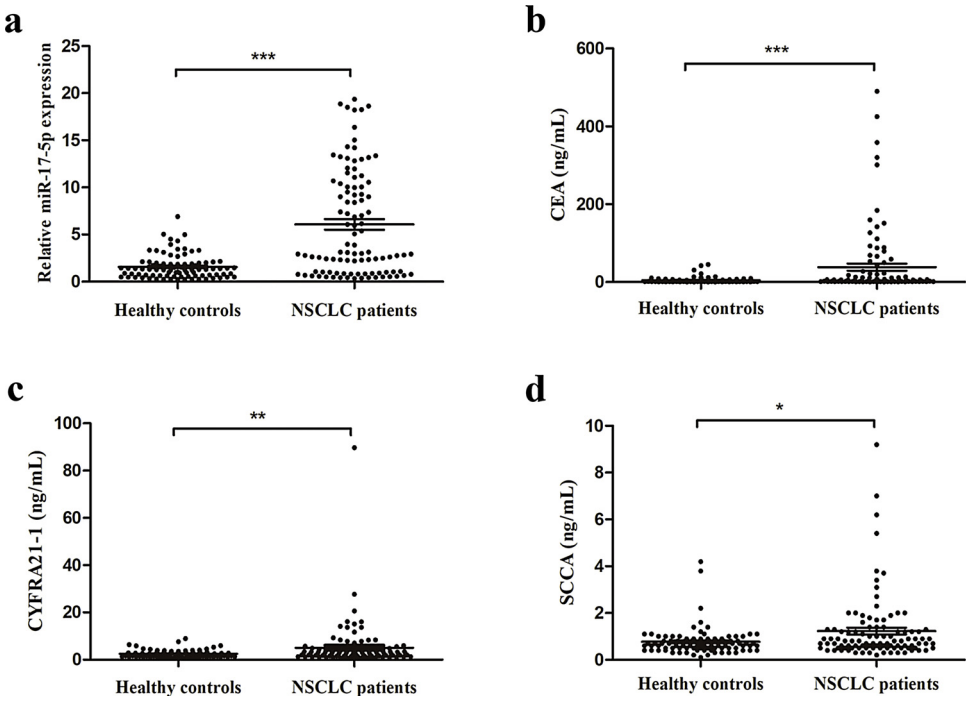

Fig. 2. Expression levels of exosomal miR-17-5p, CEA, CYFRA21-1 and SCCA in serum samples of NSCLC patients and healthy controls in the training set. (a) Exosomal miR-17-5p was up-regulated in the serum of NSCLC patients compared to healthy controls. (b–d) CEA, CYFRA21-1 and SCCA were over-expressed in NSCLC patients compared with healthy controls. * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$.

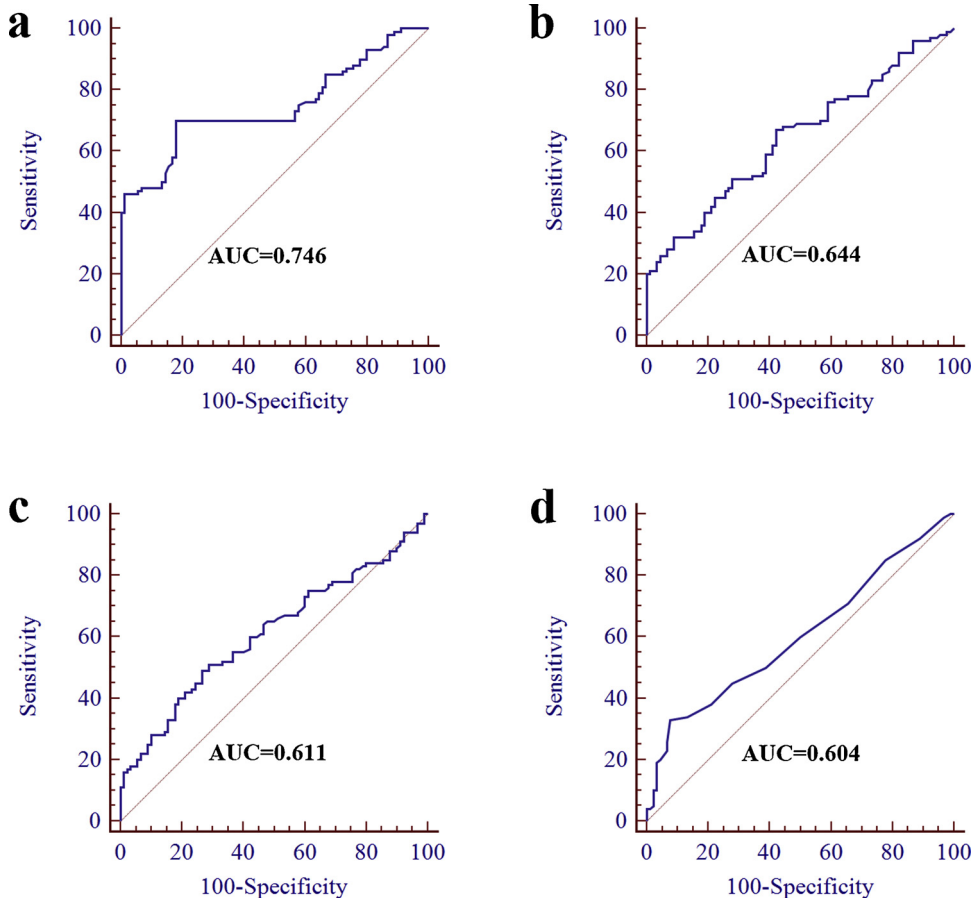

Fig. 3. ROC analyses of exosomal miR-17-5p, CEA, CYFRA21-1 and SCCA in the training set for diagnosis of NSCLC. The AUC of exosomal miR-17-5p (a) was greater than that of CEA (b), CYFRA21-1 (c) and SCCA (d).

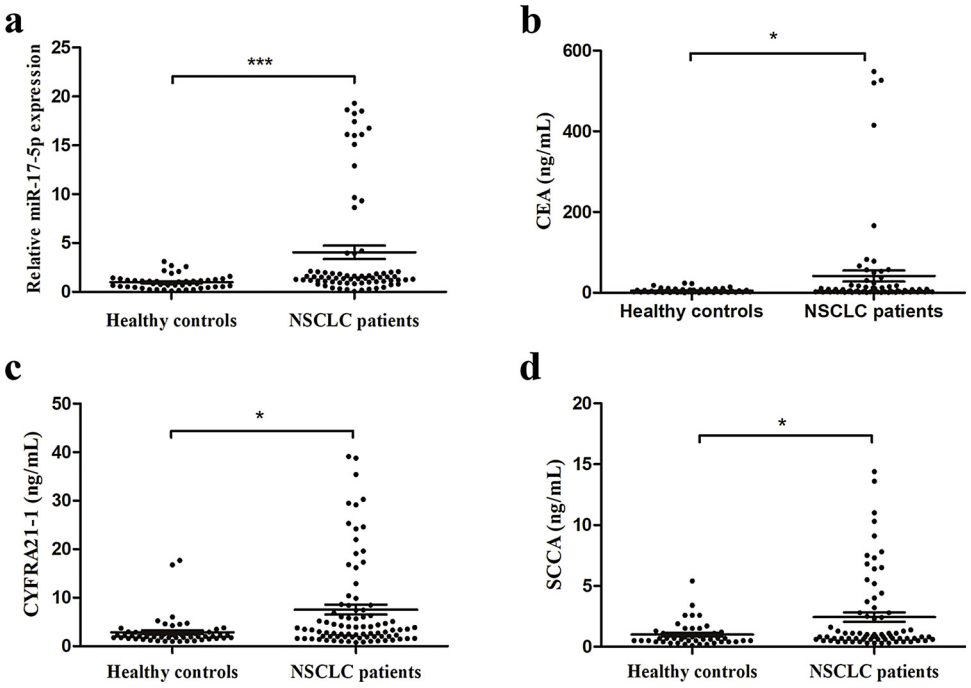

Fig. 4. Expression levels of exosomal miR-17-5p, CEA, CYFRA21-1 and SCCA in serum samples of NSCLC patients and healthy controls in the validation set. (a) Exosomal miR-17-5p was up-regulated in the serum of NSCLC patients compared to healthy controls. (b–d) CEA, CYFRA21-1 and SCCA were over-expressed in NSCLC patients compared with healthy controls. * represents $P < 0.05$, *** represents $P < 0.001$.

healthy controls from the training set) and were eliminated in the subsequent study (Table 2). Meanwhile, the expression levels of three clinical commonly-used tumor markers (CEA, CYFRA21-1 and SCCA) were evaluated in the training set (Fig. 2b–d). The diagnostic accuracy of miR-17-5p measured by AUC was 0.746 (95% CI = 0.677 to 0.806, sensitivity = 70.0% and specificity = 82.2%, Fig. 3a). In addition, the AUCs of CEA, CYFRA21-1 and SCCA were 0.644 (95% CI = 0.571 to 0.712, sensitivity = 67.0% and specificity = 57.8%, Fig. 3b), 0.611 (95% CI = 0.538 to 0.681, sensitivity = 49.0% and specificity = 73.3%, Fig. 3c) and 0.604 (95% CI = 0.530 to 0.674, sensitivity = 33.0% and specificity = 92.2%, Fig. 3d), respectively.

3.3. Validation of the up-regulated expression of SE-derived miR-17-5p in NSCLC

qRT-PCR was performed in another independent validation set (including 72 NSCLC patients and 47 healthy controls) to further verify the up-regulated expression of miR-17-5p. No significant differences were observed in the distribution of age, sex and tumor characteristics for the NSCLC and healthy controls between the training and validation sets (Table 1). Result showed that the dysregulated expression trend was consistent between the training set and the validation set (Fig. 4a). In addition, the expressions of CEA, CYFRA21-1 and SCCA in the validation set are shown in Fig. 4b–d. The corresponding AUCs of miR-17-5p, CEA, CYFRA21-1 and SCCA were 0.738 (95% CI = 0.649 to 0.814, sensitivity = 66.7% and specificity = 76.6%, Fig. 5a), 0.617 (95% CI = 0.523 to 0.704, sensitivity = 26.4% and specificity = 93.6%, Fig. 5b), 0.684 (95% CI = 0.593 to 0.776, sensitivity = 59.7% and specificity = 78.7%, Fig. 5c) and 0.634 (95% CI = 0.541 to 0.721, sensitivity = 26.4% and specificity = 95.7%, Fig. 5d), respectively.

3.4. Construction of the SE-derived miRNA panel for NSCLC diagnosis

The diagnostic formula was as follows: Logit (P) = 0.6281 – miR-17-5p × 0.0959 – CEA × 0.0027 – CYFRA21-1 × 0.0170 – SCCA × 0.1799. ROC analysis was used to evaluate the diagnostic efficacy of 4-molecule panel in the training set, and the AUC of the panel was 0.860 (95% CI = 0.802 to 0.906, sensitivity = 63.0% and specificity = 93.3%, Fig. 6a), which was significantly higher than that of any molecule alone (all at $P < 0.05$).

3.5. Validation of the diagnostic performance of the 4-molecule panel

We further verified the diagnostic performance of the 4-molecule panel in the validation set (including 72 NSCLC patients and 47 healthy controls). The results of ROC analysis showed that the AUC of the 4-molecule panel was 0.844 (95% CI = 0.766 to 0.904, sensitivity = 76.4% and specificity = 76.6%, Fig. 6b).

3.6. Correlation between expression of miR-17-5p and clinicopathological features of patients with NSCLC

To investigate whether the expression of miR-17-5p is associated with the clinicopathological features of NSCLC patients, we organized and analyzed data of the clinical-demographic characteristics of patients with NSCLC at the validation stage. Statistical analysis demonstrated that higher expression of miR-17-5p was remarkably correlated with lymph node metastasis ($P < 0.05$, Table 3). However, we did not find any significant association between miR-17-5p and age, sex, tumor size or tumor stage (all at $P > 0.05$, Table 3).

4. Discussion

Exosomes are produced through the endocytic pathway and excreted from the cytoplasmic membrane into the extracellular circumstance via multivesicular bodies [24]. Simultaneously, some specific membrane proteins, such as CD9 and TSG101 are recognized as markers for identifying true exosomes. Our study showed the classic cup-shape of exosomes by TEM; the size distribution and concentration of exosomes detected by NTA was in the scope of exosomes and consistent with the exosomal particle size distribution; the specific markers CD9 and TSG101 proteins on the membrane of exosomes were also detected by western blotting. Collectively, all the above results indicated that exosomes were triumphantly extracted from the serum specimens, which laid a foundation for further study of exosomal biomarkers.

Exosomes released by tumor cells frequently carry proteins, lipids and nucleic acids to exchange essential information between local and distant recipient cells. Since exosomes can be easily acquired and

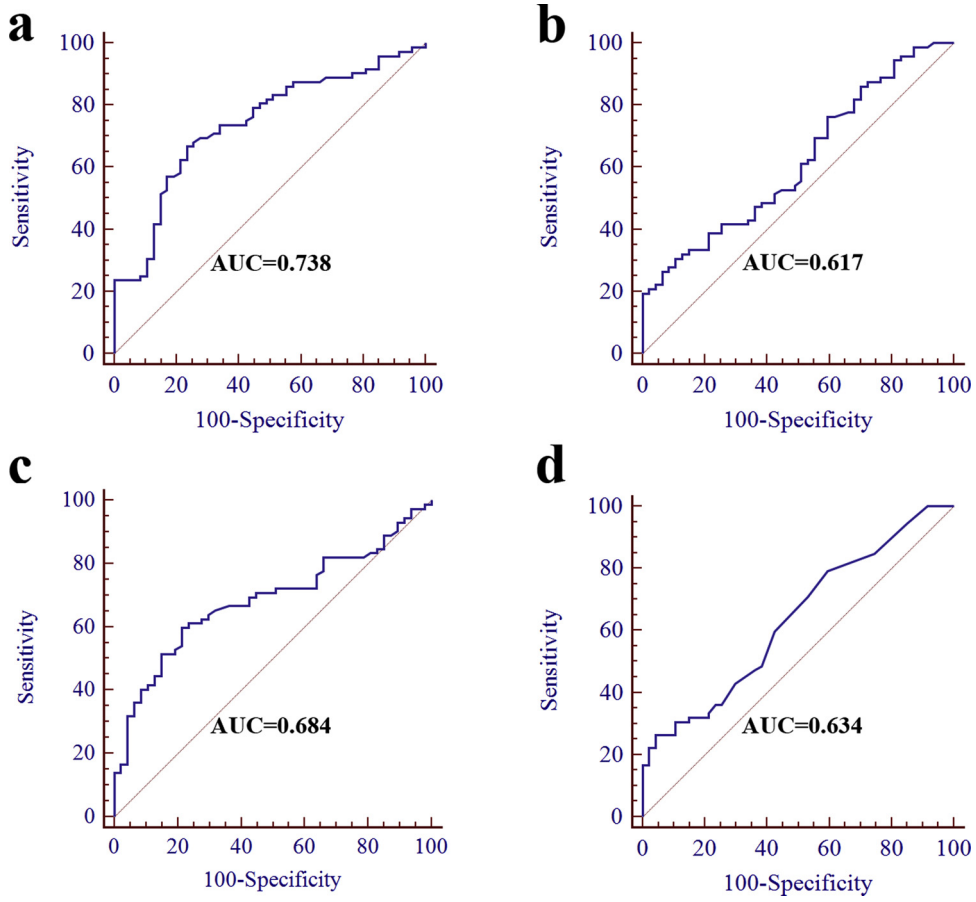

Fig. 5. ROC analyses of exosomal miR-17-5p, CEA, CYFRA21-1 and SCCA in the validation set for diagnosis of NSCLC. The AUC of exosomal miR-17-5p (a) was greater than that of CEA (b), CYFRA21-1 (c) and SCCA (d).

characterized from most body fluids, then they may serve as prospective "liquid biopsy" biomarkers for lung cancer [25–28]. While exosome carries miRNA circulating in blood, the uppermost ribonucleic acids in exosome is well protected by phospholipid membrane from being degraded by ribonuclease [29]. This strengthened the promising potency of exosome-derived miRNAs to act as biomarkers for cancer diagnosis, progression, therapy response and so on [30,31]. In the present study, we focused on the exosome-derived cluster of miR-17-92 to profile its each member, and the results demonstrated that miR-17-5p expression was significantly up-regulated in NSCLC patients than the healthy controls for the first time, indicating that miR-17-5p might be a potential diagnostic biomarker for NSCLC patients.

NSCLC is a heterogeneous malignancy with disparate driving factors, and most of the specific driving mechanism remains unknown. The diversity in expression levels of exosome encapsulated proteins and nucleic acids might reflect this heterogeneity. Given that no specific and efficient biomarker for NSCLC diagnosis is in clinical application at present, then combined detection of tumor markers can improve the accuracy of diagnosis, we initially introduced three NSCLC markers (CEA, CYFRA21-1 and SCCA) combined with exosomal miR-17-5p together to establish a 4-molecule diagnostic panel, the results of multivariate logistic regression model showed an optimal diagnostic

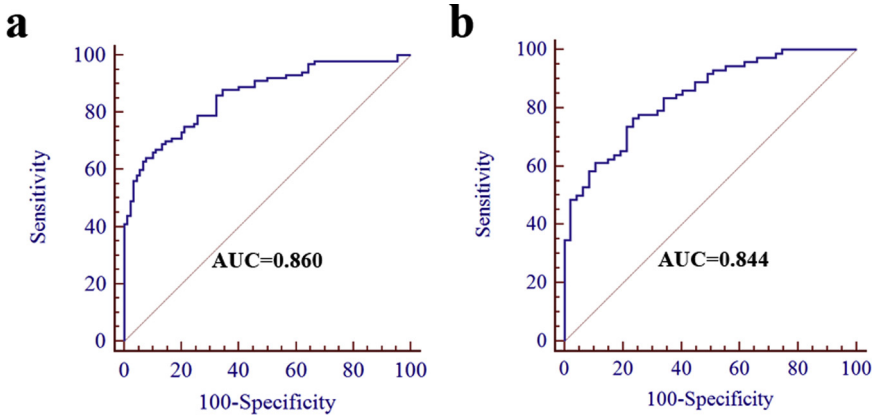

Fig. 6. Evaluation the diagnostic performance of 4-molecular panel for NSCLC. ROC analysis was used to evaluate the performance of 4-molecular panel for the diagnosis of NSCLC in the training set (a) and validation set (b), respectively.

Table 3
Correlation between concentration of miR-17-5p and clinicopathological characteristics of NSCLC patients in the validation set [median (interquartile range)].

Parameters	Total cases	miR-17-5p	*P* Value
Age (years)			> 0.05
≤ 63	38	1.353 (0.902-1.902)	
> 63	34	1.580 (1.167-8.805)	
Sex			> 0.05
Male	43	1.464 (0.9461-4.199)	
Female	29	1.505 (1.088-1.926)	
Tumor size (diameter)			> 0.05
≤3cm	34	1.459 (0.9396-1.89)	
> 3cm	38	1.485 (1.102-5.308)	
Lymph node metastasis			< 0.05
Negative	49	1.366 (0.9671-1.84)	
Positive	23	1.972 (1.275-9.318)	
Tumor stage			> 0.05
I	29	1.395 (0.8634-1.743)	
II	27	1.464 (1.102-4.199)	
III	16	1.752 (1.264-9.147)	

NSCLC: non-small cell lung cancer.

efficiency and might serve as a novel diagnostic indicator for NSCLC clinically.

Although this is the first study identifying the up-regulated expression of exosomal miR-17-5p and further established a 4-molecule diagnostic panel for NSCLC patients, meanwhile, we are also aware of some limitations of this study. NSCLC includes different pathological subtypes, Jin X et al has identified tumor-derived exosomal miRNAs that are able to discriminate between adenocarcinoma (Ad) and squamous cell carcinoma (SCC) by next-generation sequencing for early NSCLC diagnosis [22]. In the present study, we have previously analyzed the differential expression of miR-17-5p in Ad and SCC in 172 patients with NSCLC. However, the analysis showed that there was no significant difference in the expression of miR-17-5p between Ad and SCC. Considering that the sample size of patients with SCC is relatively small, and there were more Ads, comparisons with this would result in some bias. To further investigate the differential expression of miR-17-5p in Ad and SCC, a larger sample size is needed, and we will validate it in the upcoming large-scale multicenter study. In addition, we will systematically detect the different expression of miRNAs signature combined with biochemical markers for particular subtypes of NSCLC (especially for Ad) in the future.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgement

This work was supported by the National Natural Science Foundation of China [grant numbers 81400029, 81301832].

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152466.

References

[1] W.D. Travis, N. Rekhtman, Pathological diagnosis and classification of lung cancer in small biopsies and cytology: strategic management of tissue for molecular testing, Semin. Respir. Crit. Care Med. 32 (2011) 22–31.
[2] L. Osmani, F. Askin, E. Gabrielson, et al., Current WHO guidelines and the critical role of immunohistochemical markers in the subclassification of non-small cell lung carcinoma (NSCLC): Moving from targeted therapy to immunotherapy, Semin. Cancer Biol. 52 (2018) 103–109.
[3] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2019, CA Cancer J. Clin. 69 (2019) 7–34.
[4] K.A. Cronin, A.J. Lake, S. Scott, et al., Annual report to the nation on the status of cancer, part I: national cancer statistics, Cancer 124 (2018) 2785–2800.
[5] G.F. Coutinho, R. Pancas, E. Magalhães, et al., Diagnostic value of surgical lung biopsy: comparison with clinical and radiological diagnosis, Eur. J. Cardiothorac. Surg. 33 (2008) 781–785.
[6] L.T. Tanoue, N.T. Tanner, M.K. Gould, et al., Lung cancer screening, Am. J. Respir. Crit. Care Med. 191 (2015) 19–33.
[7] H. I, J.Y. Cho, Lung Cancer biomarkers, Adv. Clin. Chem. 72 (2015) 107–170.
[8] A. Grimaldi, M.R. Zarone, C. Irace, et al., Non-coding RNAs as a new dawn in tumor diagnosis, Semin. Cell Dev. Biol. 78 (2018) 37–50.
[9] E. Andrés-León, I. Cases, S. Alonso, et al., Novel miRNA-mRNA interactions conserved in essential cancer pathways, Sci. Rep. 7 (2017) 46101.
[10] H. Ma, J.S. Pan, L.X. Jin, et al., MicroRNA-17˜92 inhibits colorectal cancer progression by targeting angiogenesis, Cancer Lett. 376 (2016) 293–302.
[11] Y. Li, M. Lauriola, D. Kim, et al., Adenomatous polyposis coli (APC) regulates miR17-92 cluster through β-catenin pathway in colorectal cancer, Oncogene 35 (2016) 4558–4568.
[12] H. Zhu, C. Han, T. Wu, MiR-17-92 cluster promotes hepatocarcinogenesis, Carcinogenesis 36 (2015) 1213–1222.
[13] C. Liang, X. Zhang, H.M. Wang, et al., MicroRNA-18a-5p functions as an oncogene by directly targeting IRF2 in lung cancer, Cell Death Dis. 8 (2017) e2764.
[14] L. Milane, A. Singh, G. Mattheolabakis, et al., Exosome mediated communication within the tumor microenvironment, J. Control. Release 219 (2015) 278–294.
[15] Q. Fan, L. Yang, X. Zhang, et al., The emerging role of exosome-derived non-coding RNAs in cancer biology, Cancer Lett. 414 (2018) 107–115.
[16] T. Gracia, X. Wang, Y. Su, et al., Urinary exosomes contain MicroRNAs capable of paracrine modulation of tubular transporters in kidney, Sci. Rep. 7 (2017) 40601.
[17] T. Matsumura, K. Sugimachi, H. Iinuma, et al., Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer, Br. J. Cancer 113 (2015) 275–281.
[18] D.P. Joyce, M.J. Kerin, R.M. Dwyer, Exosome-encapsulated microRNAs as circulating biomarkers for breast cancer, Int. J. Cancer 139 (2016) 1443–1448.
[19] Z. Zhao, Y. Yang, Y. Zeng, et al., A microfluidic ExoSearch chip for multiplexed exosome detection towards blood-based ovarian cancer diagnosis, Lab. Chip 16 (2016) 489–496.
[20] S. Ambs, R.L. Prueitt, M. Yi, et al., Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer, Cancer Res. 68 (2008) 6162–6170.
[21] G. Rabinowits, C. Gerçel-Taylor, J.M. Day, et al., Exosomal microRNA: a diagnostic marker for lung cancer, Clin. Lung Cancer 10 (2009) 42–46.
[22] X. Jin, Y. Chen, H. Chen, et al., Evaluation of Tumor-Derived Exosomal miRNA as Potential Diagnostic Biomarkers for Early-Stage Non-Small Cell Lung Cancer Using Next-Generation Sequencing, Clin. Cancer Res. 23 (2017) 5311–5319.
[23] F.C. Detterbeck, K. Chansky, P. Groome, et al., IASLC staging and prognostic factors committee, advisory boards, and participating institutions, the IASLC lung cancer staging project: methodology and validation used in the development of proposals for revision of the stage classification of NSCLC in the forthcoming (Eighth) edition of the TNM classification of lung cancer, J. Thorac. Oncol. 11 (2016) 1433–1446.
[24] C.F. Ruivo, B. Adem, M. Silva, et al., The Biology of Cancer Exosomes: Insights and New Perspectives, Cancer Res. 77 (2017) 6480–6488.
[25] S. Cui, Z. Cheng, W. Qin, et al., Exosomes as a liquid biopsy for lung cancer, Lung. Cancer. 116 (2018) 46–54.
[26] I. Vanni, A. Alama, F. Grossi, et al., Exosomes: a new horizon in lung cancer, Drug Discov. Today 22 (2017) 927–936.
[27] B. Sandfeld-Paulsen, N. Aggerholm-Pedersen, R. Bæk, et al., Exosomal proteins as prognostic biomarkers in non-small cell lung cancer, Mol. Oncol. 10 (2016) 1595–1602.
[28] Y. Sun, C. Huo, Z. Qiao, et al., Comparative Proteomic Analysis of Exosomes and Microvesicles in Human Saliva for Lung Cancer, J. Proteome Res. 17 (2018) 1101–1107.
[29] T.B. Steinbichler, J. Dudás, H. Riechelmann, et al., The role of exosomes in cancer metastasis, Semin. Cancer Biol. 44 (2017) 170–181.
[30] B.N. Hannafon, Y.D. Trigoso, C.L. Calloway, et al., Plasma exosome microRNAs are indicative of breast cancer, Breast Cancer Res. 18 (2016) 90.
[31] B. Malla, D.M. Aebersold, A. Dal Pra, Protocol for serum exosomal miRNAs analysis in prostate cancer patients treated with radiotherapy, J. Transl. Med. 16 (2018) 223.