Tumor-derived exosomal miR-155-5p and miR-658 in serum as diagnostic biomarkers for early-stage non-small cell lung cancer

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Research

Keywords: Non-small cell lung cancer, Serum, Exosome, MiRNAs, Diagnostic biomarkers

Posted Date: July 9th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-40703/v1>

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Abstract

Background

Exosomal microRNAs (ExmiRNAs) provided a non-invasive and ideal method for cancer diagnosis. However, few studies identified the role of specific serum ExmiRNAs profiles in early non-small cell lung cancer (NSCLC) diagnosis, especially for 0 and I stage. Herein, the present study was designed to validate the novel serum ExmiRNAs as diagnostic biomarkers for early-stage NSCLC.

Methods

Serum exosomes were collected from the healthy donors and NSCLC patients by ultracentrifugation, and characterized with qNano, TEM, and western immunoblotting. Exosomal RNAs were subjected to miRNA array for evaluating the expression levels of miRNAs. Partly differently expressed serum exosomal miRNAs were verified by large-scale samples from 312 healthy donors and 318 NSCLC patients.

Results

The expression levels of ExmiR-155-5p and ExmiR-658 were significantly down-regulated in NSCLC patients compared to healthy donors ($p < 0.0001$ and $p < 0.0001$, respectively), and they could differentiate NSCLC patients from healthy donors with area under the ROC curve (AUC) of 0.716 and 0.728, respectively. In addition, combination of ExmiR-155-5p and ExmiR-658 could improve the diagnostic capacity with AUC value of 0.781. Moreover, ExmiR-155-5p and ExmiR-658 could differentiate early-stage NSCLC patients (0 and I stage) from healthy donors with AUC values of 0.668 and 0.735, respectively, combination could improve the diagnostic capacity with AUC value of 0.759. Specifically, the expression of ExmiR-155-5p was significantly decreased in patients with lymph node metastasis and distant metastasis ($p = 0.0018$ and $p = 0.0077$, respectively).

Conclusions

Our results identified that serum ExmiR-155-5p and ExmiR-658 were promising diagnostic biomarkers for early-stage NSCLC, and combination of them could improve the diagnostic capacity.

Background

Lung cancer is a leading cause closely, associated with cancer deaths in the world, with more than 1.4 million deaths every year [1, 2]. According to histological type, lung cancer is mainly composed by small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), which occupies about 85% of lung cancer cases [3]. Despite advances in the diagnosis and novel therapies of NSCLC, the overall 5-year survival rate still remains less than 20% [4]. Besides, the main reason for high mortality of NSCLC is that

NSCLC patients present with advanced stage at diagnosis, and have missed the best opportunity of accepting operation. If NSCLC patients can be found and diagnosed in early stage, there will be more treatment options to ensure better overall survival. Liquid biopsy is a currently promising technology for early diagnosis of NSCLC based on blood contents, such as circulating tumor cells [5], circulating tumor DNA [6], and exosomes [7].

Exosomes are known as small membrane vesicles, with a diameter of 30–100 nm, which can be released by most types of cells, including cancer cells [8, 9], and contain heterogeneous genetic materials, such as proteins, lipids, DNAs, non-coding RNAs (long non-coding RNAs, microRNAs), and other small molecules. Increasing evidences revealed that exosomes, as the intercellular communication media, can be delivered to target cells [10, 11]. Moreover, after being absorbed, exosomes can significantly change the biological behaviors of recipient cells with altering tumor microenvironment and facilitating epithelial-to-mesenchymal transition (EMT) progress, further leading to tumor occurrence, invasion and metastasis. Furthermore, emerging evidence has demonstrated that exosomes, especially exosome-delivered miRNAs, could be used as promising noninvasive diagnostic and / or prognostic biomarkers in cancer detection [12–14].

MiRNAs are short, single-stranded and relatively conservative RNA molecules with about 22 nucleotides in length [15, 16], representing a class of small nonprotein coding RNAs. MiRNAs, parceled in exosomes, are considered to be stabilized from RNase and highly enriched contrasted to the plasms and serum [17, 18]. Given that miRNAs are related to the origination and progression of different diseases such as cancers [10], and are dysregulated in several categories of cancer patients. So exosomal miRNAs can be used as molecular biomarkers for cancer diagnosis [19, 20]. For instance, plasma exosomal miR-141-3p and miR-375 were significantly over-expressed in rectal cancer patients with synchronous liver metastasis, and can be employed as diagnostic markers of rectal cancer aggressiveness and systemic dissemination [21]. In addition, the expressions of serum exosomal miR-99b-5p and miR-150-5p were obviously decreased in colorectal cancer patients, and these two serum exosomal miRNAs could act as critical biomarkers for diagnosing colorectal cancer patients [22].

In our study, we evaluated differential serum exosomal miRNAs profile by miRNA array between NSCLC patients and healthy donors. Consequently, serum exosomal miR-155-5p and miR-658 were selected, and their diagnostic potential and relationship with clinical characteristics for early-stage NSCLC were analyzed. Thus, these serum exosomal miRNAs could be served as novel diagnostic biomarkers for NSCLC, especially for early-stage NSCLC.

Materials And Methods

Patients and samples

A total of 318 NSCLC patients were recruited between March 2019 to July 2019 from Shandong Cancer Hospital, Affiliated to Shandong First Medical University (Jinan, China). None of NSCLC patients have received treatment, including surgery, radiotherapy and chemotherapy. Clinical and pathological

characteristics of patients, including age, gender, smoking, TNM stage, (according to the eighth edition American Joint Committee on Cancer) were obtained from the patients' records. In addition, a total of 312 healthy donors, who did not have any other tumor disease, were acquired from the same hospital. Exosomes from 5 healthy donors and 10 NSCLC patients were subjected to miRNA array, and then 318 patients and 312 controls were subjected to verification. The peripheral blood from patients and healthy donors were collected and then RT-PCR centrifuged at 1,000× g for 10 min at 4°C. The serums were then transferred to fresh tubes and stored at -80°C for exosome isolation.

Isolation of Exosomes

Serum exosomes were isolated using ultracentrifugation according to the procedures as described previously [20, 23]. Briefly, the serum was thawed on ice and centrifuged at 10,000×g for 30 min at 4°C to remove the cellular debris. Then 1 mL of supernatant was ultracentrifuged (Beckman Coulter, Brea, CA, USA) at 100,000× g for 2 h at 4°C to pelletize exosomes. Exosome pellets were characterized with qNano, TEM, and western immunoblotting, followed by miRNA sequencing and RNAs extraction.

qNano assay

The size and distribution of exosomes isolated from serum were analyzed using the Tunable Resistive Pulse Sensing (TRPS) on the qNano (Izon Science Ltd, Christchurch, New Zealand) according to the manufacturer's instructions. Data were analyzed by software (Izon Control Suite version 3.3.2.2001; Izon Science).

Transmission Electron Microscopy (TEM) assay

15 µL of exosome samples were added on formvar-coated copper grid. After 1 min, the remaining liquid was removed by filter paper and the samples were placed in 15µL of 2% uranyl acetate staining solution for 1 min at room temperature. Subsequently, excess liquid was wiped off, and the copper grid was baked under the lamp for 10 min, followed by observing the exosomes by FEI Tecnai T20 transmission electron microscope (FEI Company, USA).

Western immunoblotting

Exosomes or cellular proteins were separated using 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocked with 5% evaporated skimmed milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h, the membranes were probed with rabbit primary antibodies against CD63, TSG101, and GM130 overnight at 4°C. Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The immunoreactive bands were visualized by ECL blotting detection reagents (Bio-Rad, USA).

MiRNA profiling and Data Analysis

Qualified RNA from exosomes was used to label miRNA by the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark) according to the manufacturer's instruction. The Hy3™-labeled samples were hybridized on to the miRCURY™ LNA Array (v.19.0) (Exiqon) according to the instruction manual. Afterwards, the wash buffer kit (Exiqon) was used to wash the slides for several times, followed by scanning with the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). Images were uploaded into GenePix Pro 6.0 software (Axon) for extracting data and aligning the grid. MiRNAs, with intensities ≥ 30 , were selected to calculate the normalization factor. After normalizing the expressed data, miRNAs, those were significant differentially expressed by fold change ≥ 2.0 , were filtered. MiRNA expression levels between the samples were distinguished by hierarchical clustering.

RNA isolation and Real-Time PCR (RT-PCR)

All processes were carried out in an RNase-free area. Total RNAs were isolated from serum exosomes using TRIzol reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, the Mix-X miRNA First Strand Synthesis Kit (TaKaRa Bio, Nojihigashi, Kusatsu, Japan) was used to reverse-transcribe RNA into complementary DNA (cDNA) according to the manufacturer's protocol. RT-PCR was performed using TB-Green Premix Ex Taq II Reagent (TaKaRa Bio) according to the manufacturer's instructions on a Roche LightCycler480 System (Roche Diagnostics, Basel, Switzerland) [22, 24]. The relative expression levels of miRNAs were calculated using the following equation: $\Delta CT = Ct_{miRNA} - Ct_{U6}$ as described previously [25]. Each sample was analyzed in duplicate.

Statistical Analysis

SPSS 22.0 (IBM, Ehningen, Germany) and GraphPad Prism 6.0 (San Diego, CA, USA) were used for statistical analysis. The different expressions of miRNAs among groups were determined using the Mann-Whitney unpaired test or paired t test. The association between miRNAs and the clinical characteristics was evaluated by χ^2 test or paired-samples t test. Receiver operating characteristic (ROC) and area under the curve (AUC) were applied to assess the diagnostic power of the candidate predictors. $p < 0.05$ was considered to be statistically significant.

Results

Identification of isolated serum exosomes

Serum exosomes, isolated from NSCLC patients and healthy donors by ultracentrifugation, were characterized by qNano, TEM and immunoblotting. As shown in Figure 1A, qNano analysis identified that the size of isolated serum exosomes distributed from approximately 50 nm to 150 nm in diameter. This was in agreement with the results of TEM, that isolated serum exosomes had a spherical morphology with a diameter of 50-150 nm as indicated in Figure 1B. In addition, western immunoblotting confirmed the two well-known protein markers, including CD63 and TSG101 [19, 22], which were enriched in exosomes but absent in cell extracts (Figure 1C). Besides, GM130 as a negative control was only detected

in the cell extracts but not in exosomes [20]. These data demonstrated that the vesicles isolated from serum by ultracentrifugation were truly exosomes.

Exosomal miRNA Profile

Serum exosomes from 5 healthy donors and 10 NSCLC patients were subjected to RNA isolation and miRNA sequencing, the results of raw exosomal miRNA expression profile showed that a total of 1,937 differential has-miRNAs were screened, and 22 miRNAs (10 upregulated and 12 downregulated, Tables 1) were selected based on >2.0-fold difference between the healthy donors and NSCLC patients (Figure 2).

Serum exosomal miR-155-5p and miR-658 as diagnostic biomarkers for NSCLC patients

To identify the potential serum exosomal miRNA biomarkers in NSCLC, RT-PCR was performed to validate the abnormally expressed serum exosomal miRNAs for large-scale between 312 healthy donors and 318 NSCLC patients. As shown in Figure 3A and 3B, only serum exosomal miR-155-5p and miR-658 can distinguish NSCLC patients from healthy controls with obviously down-regulated expressions ($p < 0.0001$ and $p < 0.0001$, respectively). Moreover, ROC curve analysis was employed to assess the diagnostic power of serum exosomal miR-155-5p and miR-658. When compared with the healthy donors, the ROC curves from the NSCLC patients showed that serum exosomal miR-155-5p and miR-658 revealed a classifier with AUC values of 0.728 (95% CI: 0.690-0.767) and 0.716 (95% CI: 0.676-0.755), respectively (Figure 3C and 3D). Furthermore, combination of serum exosomal miR-155-5p and miR-658 improved diagnostic capacity with AUC values of 0.781 (95% CI: 0.746-0.816) shown in Figure 3 E. Besides, the expression levels of serum exosomal miR-155-5p and miR-658 have been found to be closely correlated with early-stage NSCLC patients (0 and I stage) compared to healthy donors ($p < 0.0001$ and $p < 0.0001$, respectively) (Figure 4A and 4B). Serum exosomal miR-155-5p isolated from early-stage NSCLC patients presented the diagnostic capacity with AUC of 0.668 (95% CI: 0.611-0.726) compared to the healthy donors (Figure 4C). Moreover, when relative to the healthy donors, the ROC curves from the early-stage NSCLC patients showed that serum exosomal miR-658 revealed a classifier with an AUC of 0.735 (95% CI: 0.682-0.788) (Figure 4D). Finally, combination of serum exosomal miR-155-5p and miR-658 could greatly improve the diagnostic efficiency of early-stage NSCLC (AUC = 0.759, 95% CI: 0.706-0.813) (Figure 4E).

Correlations between serum exosomal miRNAs and clinical pathology characteristics

The relationship between serum exosomal miRNAs and the clinical pathology characteristics of NSCLC patients were conducted. As shown in Table 2, the results demonstrated that both serum exosomal miR-155-5p and miR-658 were not correlated with gender, age, smoking, drinking and histological type. Interestingly, serum exosomal miR-155-5p can segregate NSCLC patients with metastasis from patients without metastasis, and expression levels of serum exosomal miR-155-5p were significantly decreased in patients with lymph node metastasis and distant metastasis ($p = 0.0018$ and $p = 0.0077$, respectively) (Figure 5A and 5B).

Diagnostic value of the combination panel of serum exosomal miRNAs with conventional biomarkers

Conventional biomarkers, including CEA and Cyfra21-1, combined with serum exosomal miR-155-5p and miR-658 were analyzed for the diagnosis of NSCLC, and the data of Figure 6A and 6B showed that combination of serum exosomal miR-155-5p and miR-658 with CEA or Cyfra21-1 had AUC values of 0.878 (95% CI: 0.851–0.904) and 0.858 (95% CI: 0.829–0.887), respectively, higher than that for CEA or Cyfra21-1 alone. Consecutively, combination of serum exosomal miR-155-5p and miR-658 with CEA or Cyfra21-1 could greatly improve the diagnostic efficiency of early-stage NSCLC patients (0+I stage) with AUC values of 0.899 (95% CI: 0.861-0.937) or 0.867 (95% CI: 0.826-0.908), respectively, higher than that for CEA or Cyfra21-1 alone, when compared with healthy donors (Figure 6C and 6D).

Discussion

Of all new cancer diagnoses, lung cancer accounts for about 14%, and the mortality of lung cancer occupies approximately 25% of all cancer deaths [26]. In addition, survival rates of NSCLC patients decrease dramatically with the advanced progress of diagnosis. On the contrary, assuming that diagnosis for NSCLC is at an early stage, nearly 90% of mortality can be prevented [4]. Thus, it is important and urgent to establish sensitive, specific and non-invasive biomarkers for the differential diagnosis of NSCLC patients, especially for early-stage patients. Therefore, the present study was designed to confirm the vital role of serum exosomal miRNAs in diagnosis of NSCLC.

Many reports confirmed that miRNAs have better stabilization in vesicles such as exosomes, and highly enriched contrasted to the plasmas and serum [17, 18]. A study conducted by Tanaka et al. demonstrated that miR-21, stemmed from exosomes, was higher-expressed than that in serum [17]. Additionally, Takuma et al. revealed that the levels of miR-191, miR-21 and miR-451a, derived from exosomes, were significantly increased in pancreatic cancer and intraductal papillary mucinous neoplasm (IPMN) patients, while the expressions of these three miRNAs had no obvious changes among the healthy control, pancreatic cancer and IPMN groups [27]. All of these findings indicated that exosomal miRNAs, as diagnostic markers, were more suitable.

MiR-155-5p has been reported for its multiple functional roles in inflammation, immunity and especially in human malignancies [28–30]. It was found that miR-155-5p contributed to down-regulate the expression of ADME gene, drug metabolizing enzymes and transporters under the condition of liver inflammation, which was conducive to better understand and predict drug metabolism of inflammatory diseases [28]. Moreover, another study suggested that miR-155-5p were significantly increased in early stages of chronic lymphocytic leukemia (CLL), and was a modest biomarker for predicting the risk of CLL [29]. Besides, miR-155-5p also could function as a tumor suppressor factor by targeting IGF2 in Wilms tumors (WT), and up-regulation of miR-155-5p could suppress cell proliferation, migration and invasion and induce cell apoptosis [30]. Additionally, miR-155-5p was over-expressed in HPV E6/E7 mRNA positive tissues, and could increase the risk of cervical cancer. Thus, miR-155 could serve as a complement approach for diagnosing and predicting the progression of cervical cancer [31]. Moreover, exosome-mediated miR-155-5p could distinguish breast cancer patients with these patients suffering an increased risk of developing breast cancer brain metastasis, and could also enhance the chemoresistance in breast

cancer cells, providing potential novel targets for breast cancer therapy [32]. Taken together, these previous studies revealed the oncogenic features of miR-155-5p, which was beneficial to support our findings. In our study, we found that serum exosomal miR-155-5p was significantly lower-expressed, and could distinguish NSCLC patients, especially 0 and I stage NSCLC patients from healthy controls, with the AUC value of 0.716. Thus, serum exosomal miR-155-5p could be a potential diagnostic biomarker for early-stage NSCLC patients. Interestingly, serum exosomal miR-155-5p was also a promising non-invasive diagnostic marker for distinguishing metastatic NSCLC patients from non-metastatic NSCLC patients.

At present, there are few studies on the miR-658. In previous studies, miR-658 was down-regulated in gastric cancer with no metastasis compared with gastric cancer with distant metastasis. Further, elevated miR-658 facilitated the migration and invasion of gastric cells, and was closely associated with of gastric cancer with no metastasis by activating PAX3 [33]. Moreover, another study revealed that exosomal miR-658 derived from lung cancer cells with gefitinib-resistant could convert sensitive cells into a resistant phenotype, providing a novel mechanism and target for therapy in cancer patients suffering with resistance against gefitinib [34]. In sum, these previous findings testified that miR-658 played an essential role in human cancers and sustained our present research. Our study demonstrated that serum exosomal miR-658 were significantly down-regulated, and could also distinguish NSCLC patients, especially 0 and I stage NSCLC patients from healthy controls, with the AUC value of 0.735.

Serum biomarkers for diagnosis of NSCLC patients have been widely studied and applied in clinical, such as CEA, CA125, NSE, CYFRA21-1, and CA153 [19, 20]. However, there is no any effective biomarker for diagnosis of early-stage NSCLC patients. CEA and CYFRA21-1, as meaningful prognostic markers and predictors for therapeutic efficacy or metastasis and recurrence, were selected in the present study. Our data identified that CEA and CYFRA21-1 could be combined with serum exosomal miRNAs for diagnosis of early-stage NSCLC patients, and serum exosomal miR-155-5p and miR-658 combined with CEA or CYFRA21-1 could improve the diagnostic power for early-stage NSCLC patients with AUC values of 0.899 and 0.867, respectively.

To the best of our knowledge, this study was the first time to demonstrate the novel serum exosomal miR-155-5p and miR-658 as biomarkers for diagnosing NSCLC patients, and to identify serum exosomal miR-155-5p a potential non-invasive diagnostic marker for distinguishing metastasis from non-metastatic NSCLC. Our study enrolled enough specimens, which was considerable convincing. However, the present study had several limitations. On the one hand, due to lack of long-term clinical follow-up data, we could not evaluate the prognostic power of serum exosomal miR-155-5p and miR-658. On the other hand, this study focused on healthy donors and NSCLC patients, and did not include cases with begin lung diseases, such as pneumonia, pulmonary fibrosis, and so on. Thus, a further analysis should be conducted to demonstrate that these biomarkers can be employed to distinguish NSCLC patients from begin lung disease patients and healthy individuals.

Conclusion

In conclusion, down-regulation of serum exosomal miR-155-5p and miR-658 was critical for diagnosing NSCLC patients, especially with early-stage patients (0 and I stage). Combination of serum exosomal miR-155-5p and miR-658 could improve the diagnostic power. Besides, serum exosomal miR-155-5p was a potential non-invasive diagnostic marker for distinguishing metastasis from non-metastatic NSCLC.

Abbreviations

NSCLC: Non-small-cell lung cancer; TEM: Transmission Electron Microscopy; ROC: Receiver operating characteristic; AUC: Area under the curve; SCLC: small-cell lung cancer; EMT: Epithelial-to-mesenchymal transition; RT-PCR: Reverse Transcription-Polymerase Chain Reaction; TRPS: Tunable Resistive Pulse Sensing; cDNA: complementary DNA; CEA: Carcinoembryonic antigen; IPMN: Intraductal papillary mucinous neoplasm; CLL: Chronic lymphocytic leukemia.

Declarations

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of the Shandong Cancer Hospital, Affiliated to Shandong First Medical University (Jinan, China), and written informed consent was obtained from each patient.

Consent for publication

This manuscript has been seen and approved by all authors for publication.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (81773237, 81672104, 81972014), the Shandong Provincial Key Research and Development Program (2016GSF201146, 2016GSF201151, 2017GSF18183 and 2017CXGC1207). Shandong Provincial Natural Science Foundation (ZR2019MH004 and ZR2019LZL016).

Authors' Contributions

ZZJ and XRS both are responsible for the acquisition and analysis of the data and drafting of the manuscript. XRS designed the study, interpreted the data, and revised the manuscript. XGS was in charge of data analysis and manuscript revision. The rest of the co-authors all participated in the data collection, laboratory tests and revision of the manuscript.

Acknowledgements

Not applicable.

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Tables

Table 1. Part of miRNAs expression profiling data (10 upregulated and 12 downregulated)

miRNA	Fold change	Description	miRNA	Fold change	Description
Has-miR-508-5p	6.9339	Up	hsa-miR-658	0.5163	Down
hsa-miR-514a-3p	4.5196	Up	hsa-miR-92b-3p	0.2212	Down
hsa-miR-132-5p	4.2486	Up	hsa-miR-125a-5p	0.2011	Down
hsa-miR-759	3.6300	Up	hsa-miR-1301-3p	0.2004	Down
hsa-miR-514a-5p	2.6306	Up	hsa-miR-379-5p	0.1879	Down
hsa-miR-501-5p	2.6046	Up	hsa-miR-4301	0.1842	Down
hsa-miR-513b-5p	2.5420	Up	hsa-miR-221-5p	0.1619	Down
hsa-miR-369-5p	2.4585	Up	hsa-miR-130b-5p	0.1574	Down
hsa-miR-29c-5p	2.1611	Up	hsa-miR-93-5p	0.1447	Down
hsa-miR-450a-5p	1.6919	Up	hsa-miR-155-5p	0.1402	Down
hsa-miR-323a-3p	0.1368	Down	hsa-miR-128-3p	0.0980	Down

Table 2. Characteristics of NSCLC patients for differentially expressed serum exosomal miR-155-5p and miR-658

Characteristics		No. case	miR-155-5p		miR-658	
			Median with interquartile range	<i>p</i> -value	Median with interquartile range	<i>p</i> -value
Age (year)	≤62	174	6.0175 (5.3550-6.4550)	0.3757	5.1775 (3.8650-6.2150)	0.2346
	>62	144	5.8870 (5.2900-6.3900)		4.8700 (3.9750-5.7150)	
Gender	Male	191	6.0300 (5.3550-6.4750)	0.0851	5.0350 (3.9400-6.1050)	0.4131
	Female	127	5.8200 (5.3050-6.3650)		4.9450 (3.9500-5.8550)	
Histological type	SCC	75	5.9700 (5.4650-6.3050)	0.8241	5.2450 (4.6025-6.0100)	0.3912
	AD	222	5.9850 (5.3000-6.4600)		4.9725 (3.9100-5.9500)	
	others	21				
Smoking history	Yes	168	6.0125 (5.3550-6.475)	0.3062	5.0375 (3.9450-6.0825)	0.5401
	No	150	5.9050 (5.3100-6.3750)		4.9600 (3.9350-5.8550)	
Drinking history	Yes	105	5.9175 (5.3375-6.4000)	0.9578	5.0300(3.9400-6.0475)	0.6065
	No	213	5.9950 (5.3350-6.4275)		5.0250 (3.9500-5.9750)	
Lymph node metastasis	Yes	143	5.7900 (5.1600-6.3050)	0.0018	5.0550 (3.9500-5.9250)	0.4002
	No	159	6.0550 (5.5100-6.4750)		5.0250 (3.9625-6.0950)	
	unknown	16				
Distant metastasis	Yes	210	5.8250 (5.2300-6.3200)	0.0077	5.0250 (4.0450-5.9275)	0.9183
	No	94	6.1400 (5.6050-6.5550)		5.0325 (3.8600-6.2200)	
	unknown	14				

Figures

Figure 1

Figure 1

Identification of isolated serum exosomes. (A) Size distribution of exosomes ranged from 50-150 nm was analyzed by qNano system. (B) Representative data of exosomes of 50-150 nm diameter from NSCLC patients were analyzed with TEM [scale bar: 50 nm; high voltage (HV) = 80-120 kV]. (C) The exosomal protein markers, including CD63 and TSG101 were detected in serum exosomes by western immunoblotting analysis.

Figure 2

Figure 2

Exosomal miRNA Profile of NSCLC patients. Exosomal miRNA profile of the NSCLC patients. A heat map was generated after supervised hierarchical cluster analysis. The differential miRNA expression is shown in red (upregulation) vs. green (downregulation).

Figure 3

Figure 3

Serum exosomal miR-155-5p and miR-658 as diagnostic biomarkers for NSCLC patients. The expression levels of serum exosomal (A) miR-155-5p and (B) miR-658 in NSCLC patients (n =318) and healthy donors (n = 312) were evaluated by RT-PCR assay. (C) The AUC of serum exosomal miR-155-5p was 0.716 in 318 NSCLC patients and 312 healthy donors. (D) The AUC of serum exosomal miR- 658 was 0.728 in 318 NSCLC patients and 312 healthy donors. (E) The AUC of serum exosomal miR- 155-5p combined with miR-658 was 0.781 in 318 NSCLC patients and 312 healthy donors.

Figure 4

Figure 4

Serum exosomal miR-155-5p and miR-658 as diagnostic biomarkers for early-stage NSCLC patients. The expression levels of serum exosomal (A) miR-155-5p and (B) miR-658 in early-stage NSCLC patients (0 + I stage, n = 114) and healthy donors (n = 312) were evaluated by RT-PCR assay. (C) The AUC of serum exosomal miR-155-5p was 0.668 in 114 early-stage NSCLC patients and 312 healthy donors. (D) The AUC of serum exosomal miR-658 was 0.735 in 114 early-stage NSCLC patients and 312 healthy donors. (E) The AUC of serum exosomal miR-155-5p combined with miR-658 was 0.759 in 114 early-stage NSCLC patients and 312 healthy donors.

Figure 5

Figure 5

Serum exosomal miR-155-5p as a diagnostic biomarker for differentiating patients with metastatic NSCLC from patients with non-metastatic NSCLC. (A) The expression levels of serum exosomal miR-155-5p in the serum from non-lymph node metastatic (n = 143), and lymph node metastatic NSCLC patients (n = 159) were evaluated by RT-PCR assay. (B) The expression levels of serum exosomal miR-155-5p in the serum from non-distant metastasis NSCLC patients (n = 210), and distant metastasis NSCLC patients were evaluated by RT-PCR assay.

Figure 6

Figure 6

Diagnostic value of the combination panel of serum exosomal miRNAs with conventional biomarkers. (A) The AUC of serum exosomal miR-155-5p and miR-658 with CEA was 0.878 in 318 NSCLC patients and 312 healthy donors. (B) The AUC of serum exosomal miR-155-5p and miR-658 with Cyfra21-1 was 0.858 in 318 NSCLC patients and 312 healthy donors. (C) The AUC of serum exosomal miR-155-5p and miR-658 with CEA was 0.899 in 114 early-stage NSCLC patients and 312 healthy donors. (D) The AUC of serum exosomal miR-155-5p and miR-658 with Cyfra21-1 was 0.867 in 114 early-stage NSCLC patients and 312 healthy donors.