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RESEARCH

Diagnostic value of microRNA-200 expression in peripheral blood-derived extracellular vesicles in early-stage non-small cell lung cancer

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Abstract

Objective This study assessed the diagnostic value of microRNA-200 (miR-200) expression in peripheral blood-derived extracellular vesicles (EVs) in early-stage non-small cell lung cancer (NSCLC).

Methods This study retrospectively analyzed 100 healthy volunteers (the control group) receiving physical examinations, 168 early-stage NSCLC patients (the NSCLC group), and 128 patients with benign lung nodules (the benign group). The basic and clinical data of participants were obtained, including age, sex, smoking history, carbohydrate antigen 242 (CA242), carcinoembryonic antigen (CEA), carbohydrate antigen 199 (CA199), forced expiratory volume in 1 s, maximal voluntary ventilation, forced vital capacity, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and miR-200 expression. The correlation of miR-200 expression in peripheral blood-derived EVs with CA242, CEA, and CA199 was analyzed, and the diagnostic value of peripheral blood-derived EV miR-200 for early-stage NSCLC was assessed. The risk factors of early-stage NSCLC development were also determined.

Results Age, the percentage of patients with smoking history, CA242, CEA, CA199, IL-6, and TNF-α levels, and miR-200 expression in peripheral blood-derived EVs were significantly higher in the NSCLC group than in the benign and control groups. Lung disease patients with high miR-200 expression in peripheral blood-derived EVs comprised a higher percentage of patients with smoking history and mixed lesions and had higher CA242, CEA, CA199, and TNF-α levels than those with low miR-200 expression in peripheral blood-derived EVs. In lung diseases, miR-200 expression in peripheral blood-derived EVs was significantly and positively correlated with CA242, CEA, and CA199. Peripheral blood-derived EV miR-200 combined with CA242, CEA and CA199 had higher diagnostic value (area under the curve = 0.942) than single detection, along with higher specificity, and high expression of peripheral blood-derived EV miR-200 was an independent risk factor for early-stage NSCLC.

Conclusion Peripheral blood-derived EV miR-200 expression in patients with lung diseases is closely correlated with CA242, CEA, and CA199, and high expression of peripheral blood-derived EV miR-200 is an independent risk factor for early-stage NSCLC and is of high clinical diagnostic value for early-stage NSCLC.

Keywords Non-small cell lung cancer · Peripheral blood · Extracellular vesicles · MicroRNA-200 · Early stage · Diagnosis

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Introduction

Lung cancer (LC) is currently the malignant tumor with the highest mortality rate worldwide, with non-small-cell LC (NSCLC) as the most common type of LC, accounting for 85% of the total incidence of LC [8]. NSCLC is usually diagnosed at advanced-stages due to inadequate screening methods and late onset of clinical symptoms [6]. With limited treatment options and no known cure, advanced NSCLC often has short survival times and dismal prognosis [2]. Therefore, research on potential diagnostic biomarkers and therapy for early-stage NSCLC is vital for the treatment and prognosis of NSCLC patients. Serum tumor markers are common diagnostic indicators of malignant tumors, including carbohydrate antigen 242 (CA242), carcinoembryonic antigen (CEA), and carbohydrate antigen 19-9 (CA199), all of which have high clinical significance in the diagnosis of NSCLC [9, 30, 31]. Recently, frequently used tumor markers, such as CA242, CEA and CA199, are all pivotal diagnostic indicators for lung cancer [21, 30, 31], but their specificities are not high. Consequently, the diagnosis of LC requires combination with pathological examinations.

Extracellular vesicles (EVs) are a collective term for a variety of cell-derived vesicles with a membrane structure comprising exosomes and microvesicles [28]. EVs are widespread in the human body, including blood and cerebrospinal fluid, and are responsible for cell-to-cell communication [12]. EVs can carry numerous cellular components including microRNAs (miRs), mRNAs, proteins, DNA, and lipids that are transferred from donor cells to recipient cells, thus affecting several physiological and pathological events [13]. As they remain stable when encapsulated in EVs, miRs are able to spread over long distances in body fluids including blood, without degradation by extracellular nucleases, and therefore remain functionally intact in recipient cells [17]. miRs are a kind of non-coding RNA molecules implicated in cell proliferation, differentiation, and survival, which are tightly linked to the onset and progression of cancers [22]. As reported, circulating miRs are potential blood markers for the detection of early-stage cancers [24]. Of note, miR-200 is associated with the development of NSCLC [15, 19, 20, 32]. A prior study unveiled miR-200 upregulation in early-stage lung adenocarcinoma [7]. Furthermore, accumulating studies have revealed circulating exosomal miRs as a potential non-invasive diagnostic and prognostic marker of NSCLC [18, 34]. Nevertheless, no reports have yet been published on the correlation between miR-200 in peripheral blood-derived EVs and the adjunctive diagnosis and disease assessment of early-stage NSCLC patients. Therefore, this study evaluated the value of miR-200 in peripheral blood-derived EVs in the auxiliary diagnosis of early-stage NSCLC.

Materials and methods

Participants

This study retrospectively analyzed 350 patients with lung diseases (200 patients with early-stage NSCLC and 150 patients with benign lung nodules) who visited Xi'an People's Hospital (Xi'an Fourth Hospital) from January 2018 to 2023. Among patients with early-stage NSCLC, 14 patients did not meet the inclusion criteria, 13 patients matched the exclusion criteria, and 5 patients had incomplete clinical data. Eventually, 168 patients with early-stage NSCLC were included as the NSCLC group (mean age: 51.92 ± 10.26 years; 96 males and 72 females; 77 patients at stage I and 91 patients at stage II). Among patients with benign lung nodules, there were 5 patients not fulfilling the inclusion criteria, 10 patients satisfying the exclusion criteria, and 7 patients without complete clinical data. Ultimately, 128 with benign lung nodules were included as the benign group (mean age: 48.03 ± 5.49 years; 88 males and 40 females). Patients were included if matching the criteria described below: (1) patients with early-stage NSCLC who met the diagnostic criteria for NSCLC in the *Comprehensive Diagnostic and Treatment Strategies for NSCLC-NCCN Clinical Practice Guidelines in Oncology* and who were at stage I-II according to tumor-node-metastasis (TNM) staging in the newly revised criteria of the International Union Against Cancer in 1997 and the 7*th* edition of the *TNM Staging of Malignant Tumors* published in 2009; (2) patients diagnosed with benign lung nodules by pathological examinations; (3) patients who were initially diagnosed without any targeted treatment; and (4) patients aged ≥ 30 years and ≤ 80 years. Patients were excluded if satisfying the following criteria: (1) patients with dyspnea or other symptoms that may lead to hypoxia in the body; (2) patients with severe cardiac, hepatic, renal, and metabolic diseases; (3) patients with co-morbid infectious diseases; and (4) patients with a history of major surgical treatment.

In addition, 120 healthy volunteers undergoing health checkups in Xi'an People's Hospital (Xi'an Fourth Hospital) during the same period were selected, of which 4 cases failed to meet the inclusion criteria, 9 cases were excluded as per the exclusion criteria, and 7 cases had incomplete clinical data. Therefore, 100 healthy volunteers were included as the control group (mean age: 45.37 ± 5.53 years; 57 males and 43 females). Inclusion criteria for the control group are listed below: (1) age ≥ 30 years and ≤ 80 years; (2) complete clinical data; and (3) normal clinical indicators. Exclusion criteria for the control group are described below (1) a history of tumors; (2) no history of LC and no family history of LC in immediate relatives or collateral relatives within

three generations or less; (3) a history of infection in the last six months; (4) and a history of major surgical treatment.

The study adhered to ethical guidelines and norms and regulations for clinical trials in the *Declaration of Helsinki* and conformed to the Enhancing the QUAlity and Transparency of Health Research network guidelines. Additionally, the study was ratified by the Academic Ethics Committee of Xi'an People's Hospital (Xi'an Fourth Hospital).

Collection of peripheral blood samples and data

The clinical data of participants were obtained, including age, sex, smoking history, pathological type (epithelial, mesenchymal, mixed), lung function indicators (Forced Expiratory Volume in one second [FEV_1], Maximal Voluntary Ventilation [MVV], Forced Vital Capacity [FVC]), serum tumor markers (CA242, CEA, and CA199), and inflammatory indicators (interleukin-6 [IL-6] and tumor necrosis factor-α [TNF-α]). Blood samples stored in a refrigerator were harvested for enzyme-linked immunosorbent assay (ELISA) of serum indicators, and lung function indicators were examined with a spirometer (AS-407, MINATO, Osaka, Japan).

Acquisition of EVs by ultracentrifugation [3]

The plasma was attained from the refrigerator, thawed at room temperature, and centrifuged at 4 °C and 500 g for 10 min to remove impurities such as residual blood cells. After the precipitate was discarded, the supernatant was transferred to a 10 mL centrifuge tube and diluted with 9 mL of 1 × PBS. All steps of aspirating the supernatant should be careful not to blow up the precipitate. The supernatant was centrifuged with a high-speed centrifuge (OSE-HC-01, TIANGEN, Beijing, China) at 4 °C and 2000 g for 30 min. After the precipitate was removed, the supernatant was transferred to an ultracentrifuge tube and centrifuged at 4 °C and 20,000 g for 30 min with the high-speed centrifuge. After being transferred to a clean ultracentrifuge tube, the supernatant was centrifuged at 4 °C and 110,000 g for 80 min with the high-speed centrifuge. The supernatant was removed, and the ultracentrifuge tube was added with 9 mL of 1 × PBS and mixed well, followed by 80 min centrifugation at 4 °C and 110,000 g. Following supernatant removal, the EV precipitate was resuspended with 200 µL of 1 × PBS, during which air bubbles were avoided. Subsequently, the EV suspension was transferred into a 1.5 mL Eppendorf tube and preserved at − 80 °C for use.

EV characterization

The suspension of extracted EVs from peripheral blood was obtained from the − 80 °C refrigerator, melted at room temperature, and diluted tenfold. The morphological structure of EVs was observed with a transmission electron microscope (TEM; Leica, Wetzlar, Germany), and the particle size distribution and particle concentration of EVs were detected with a nanoparticle tracking analyzer (NTA; NanoSight NS300; Malvern Panalytical Ltd., Malvern, UK). Western blotting was performed to test the expression of surface antigens CD9, CD63, and TSG101 and the negative marker Calnexin in EVs. Specifically, total proteins were isolated from EVs with the Exosome Protein Extraction Kit (HR8215; BIO-LAB, Beijing, China), and the EV protein concentration was measured with the BCA kit (P0010; Beyotime, Shanghai, China). Proteins were separated by 10% SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was subjected to 2 h of sealing with 5% BSA at room temperature to block non-specific binding, followed by overnight incubation at 4 °C with primary antibodies (Abcam, Cambridge, UK) against CD9 (ab236630), CD63 (ab134045), TSG101 (ab125011), and Calnexin (ab13504). Subsequent to TBST washing, the membrane was incubated with secondary H&L-conjugated IgG antibody (ab6721, Abcam) at room temperature for 1 h and developed with ECL working solutions (EMD Millipore, Billerica, MA, USA). Some of the identified EVs were treated with RNase alone and combined with SDS Lysis Buffer, respectively, for the identification of miRs in peripheral blood-derived EVs.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Peripheral blood-derived EVs stored at − 80 °C were obtained and melted in a 37 °C water bath, and total RNA of peripheral blood-derived EVs was isolated with TRIzol reagents (abs60154; Absin, Shanghai, China), followed by the detection of the RNA concentration with an ultramicro spectrophotometer (NanDrop one; Thermo Scientific, Waltham, Massachusetts, USA). The cDNA was synthesized by reverse transcription with the miRNA qRT-PCR kit (MQPS; RiboBio, Guangzhou, China). Then, cDNA (2 µL) diluted fivefold was added to the qPCR reaction system consisting of 12.5 µL of SYBR Premix Ex Taq II, 0.5 µL of Dye II, 2 µL of 5 µM forward primer, and 1 µL of 10 µM Uni-miR RT-qPCR Primer. qRT-PCR was performed at 95 °C for 30 s, 95 °C for 5 s, and 57 °C for 34 s for 40 cycles under 7 µL ddH_2O cycling conditions [16]. The relative expression of peripheral blood-derived EV miRs was calculated using the $2^{-\Delta CT}$ method, with cel-miR-39-3p as an exogenous control gene. The utilized primers are listed in Table S1.

Statistical analysis

Data were statistically analyzed and plotted with SPSS statistical software (version 21.0; IBM, Armonk, NY, USA),

GraphPad Prism software (version 8.0.1; GraphPad Software Inc., San Diego, CA, USA), MedCalc software (version 22.2; MedCalc software Ltd., Ostend, Belgium). The Kolmogorov–Smirnov test was performed to analyze the normal distribution of data. Normally distributed measurement data, which were displayed as mean ± standard deviation, were compared between the two groups with the *t*-test and among multiple groups with one-way analysis of variance followed by Tukey's post hoc multiple comparisons test. Skewed measurement data, which were presented as quartiles, that is, median (minimum, maximum), were compared between two groups with the nonparametric test and among multiple groups with the Kruskal–Wallis rank sum test followed by Tukey's post hoc multiple comparisons test. Count data were represented by the number of cases (percentage), with the Fisher's exact test for intergroup comparisons. The relationship between miR-200 expression in peripheral blood-derived EVs and serum tumor markers (CA242, CEA, and CA199) in patients with lung nodules was analyzed with the Spearman's correlation coefficient. Receiver-operating characteristic (ROC) curves were plotted to analyze the sensitivity, specificity and the area under the ROC curve (AUC) (sensitivity refered to the proportion of judged positive samples to the actually positive samples, which was calculated as true positive/[true positive + false negative]; specificity refered to the proportion of judged negative samples to the actually negative samples, which was calculated as true negative/[true negative + false positive]). The risk of early-stage NSCLC development was analyzed with logistic regression. The test level was $\alpha = 0.05$, and *p*-values were obtained from two-sided tests. *p*-values below 0.05 were considered statistically significant.

Results

Baseline data of participants

Baseline data of participants, including age, sex, smoking history, serum tumor markers (CA242, CEA, and CA199), lung function indicators (FEV1, MVV, and FVC), and inflammatory markers (IL-6 and TNF-α) were compared among the NSCLC, benign, and control groups. The results (Table 1) displayed that the percentage of patients with smoking history, age, and CA242, CEA, CA199, IL-6, and TNF-α expression in the NSCLC group were dramatically higher than those in the benign and control groups ($p < 0.05$).

Characterization of peripheral blood-derived EVs

EVs were isolated by ultracentrifugation from peripheral blood samples of 168 patients with early-stage NSCLC,

Table 1 Comparison of general data among the NSCLC, benign, and control groups

	NSCLC (n = 168)	Benign (n = 128)	Control (n = 100)	^{a}p	^{b}p	^{c}p	*p*
Age (years)	51.92 ± 10.26	48.03 ± 5.49	45.37 ± 5.53	< 0.001	< 0.001	0.014	< 0.001
Sex (male/female)	96/72	88/40	57/43	0.053	> 0.99	0.073	0.084
Smoking history (n, %)				0.025	< 0.001	0.007	< 0.001
Yes	131 (77.98)	84 (65.63)	47 (47.00)				
No	37 (22.02)	44 (34.37)	53 (53.00)				
CA242 (U/mL)	26.84 (4.06, 45.96)	16.63 (8.02, 32.72)	17.50 (10.28, 24.79)	< 0.001	< 0.001	< 0.001	< 0.001
CEA (ng/mL)	5.32 (2.39, 13.29)	4.03 (0.36, 8.66)	3.55 (0.22, 7.90)	< 0.001	< 0.001	< 0.001	< 0.001
CA199 (U/mL)	30.64 (9.57, 51.72)	24.47 (10.10, 40.64)	16.56 (6.34, 26.87)	< 0.001	< 0.001	< 0.001	< 0.001
FEV1 (%)	84.80 ± 3.18	85.08 ± 5.04	87.30 ± 3.28	0.812	< 0.001	< 0.001	< 0.001
MVV (%)	88.14 ± 5.16	88.76 ± 5.25	92.81 ± 4.63	0.545	< 0.001	< 0.001	< 0.001
FVC (%)	80.73 ± 5.58	81.41 ± 5.99	89.54 ± 5.33	0.557	< 0.001	< 0.001	< 0.001
IL-6 (pg/mL)	68.82 (30.59, 428.39)	65.76 (36.77, 356.40)	41.24 (20.18, 62.47)	0.031	< 0.001	< 0.001	< 0.001
TNF-α (pg/mL)	106.16 (31.72, 452.11)	86.47 (30.08, 367.38)	67.62 (40.16, 95.32)	< 0.001	< 0.001	< 0.001	< 0.001

The Kolmogorov–Smirnov test was performed to analyze the normal distribution of data. Normally distributed measurement data were displayed as mean ± standard deviation and compared among multiple groups with one-way analysis of variance followed by Tukey's post hoc multiple comparisons test. Skewed measurement data were presented as quartiles, that is, median (minimum, maximum), and compared among multiple groups with the Kruskal–Wallis rank sum test followed by Tukey's post hoc multiple comparisons test. Count data for categorical variables among multiple groups were compared with the Mann–Whitney *U* test

CA242 carbohydrate antigen 242, *CEA* carcinoembryonic antigen, *CA199* carbohydrate antigen 19–9, *FEV1* forced expiratory volume in one second, *MVV* maximal voluntary ventilation, *FVC* Forced vital capacity, *IL-6* interleukin-6, *TNF-α* tumor necrosis factor-α

^{a}p: comparison between the NSCLC and benign groups

^{b}p: comparison between the NSCLC and control

^{c}p: comparison between the benign and control groups

128 patients with benign lung nodules, and 100 healthy volunteers. Under the TEM, peripheral blood-derived EVs from all participants showed a cystic bi-layered membrane structure, with typical EV morphological features (Fig. 1A). NTA analysis results exhibited that the major particle size distributions of EVs in the NSCLC, benign, and control groups were in the ranges of 30–200 nm, 25–200 nm, and 25–200 nm, respectively (Fig. 1B). Western blotting revealed positive expression of CD9, CD63, and TSG101, as well as negative expression of Calnexin in the three groups (Fig. 1C). Overall, peripheral blood-derived EVs were successfully isolated from participants.

High miR-200 expression in peripheral blood-derived EVs of patients with early-stage NSCLC

Subsequently, EVs of early-stage NSCLC patients were treated with RNase alone or combined with SDS Lysis Buffer. The obtained data demonstrated that no obvious changes were observed in miR-200 expression between untreated EVs and RNase-treated EVs ($p > 0.05$), whereas the addition of RNase combined with SDS Lysis Buffer greatly lowered miR-200 expression in EVs ($p < 0.001$) (Fig. 2A), indicating that miR-200 was encapsulated in EVs. Next, miR-200 expression in peripheral blood-derived EVs was examined with qRT-PCR. The results (Fig. 2B) disclosed that miR-200 expression in peripheral blood-derived EVs was higher in the NSCLC group (4.31 [0.90, 27.86]) than in the benign (1.89 [0.61, 5.39]) and control (0.87 [0.17, 4.89]) groups and also higher in the benign group than in the control group ($p < 0.001$).

Relationship between miR-200 expression in peripheral blood-derived EVs and clinical characteristics of patients with lung diseases

Subsequently, the median value of miR-200 expression in peripheral blood-derived EVs of patients with lung diseases (2.66) was utilized as the cut-off value, and patients were allocated into high miR-200 expression (> 2.66) and low miR-200 expression (≤ 2.66) groups. Then, we analyzed the relationship between miR-200 expression in peripheral blood-derived EVs and clinical characteristics of patients with lung diseases (age [> 50 years and ≤ 50 years], sex [male and female], smoking history [yes and no], pathological type [epithelial, mesenchymal, and mixed], serum tumor markers [CA242, CEA, and CA199], lung function indicators [FEV1, MVV, and FVC], and inflammatory indicators [IL-6 and TNF-α]). The results (Table 2) manifested no substantial differences between the high and low miR-200 expression

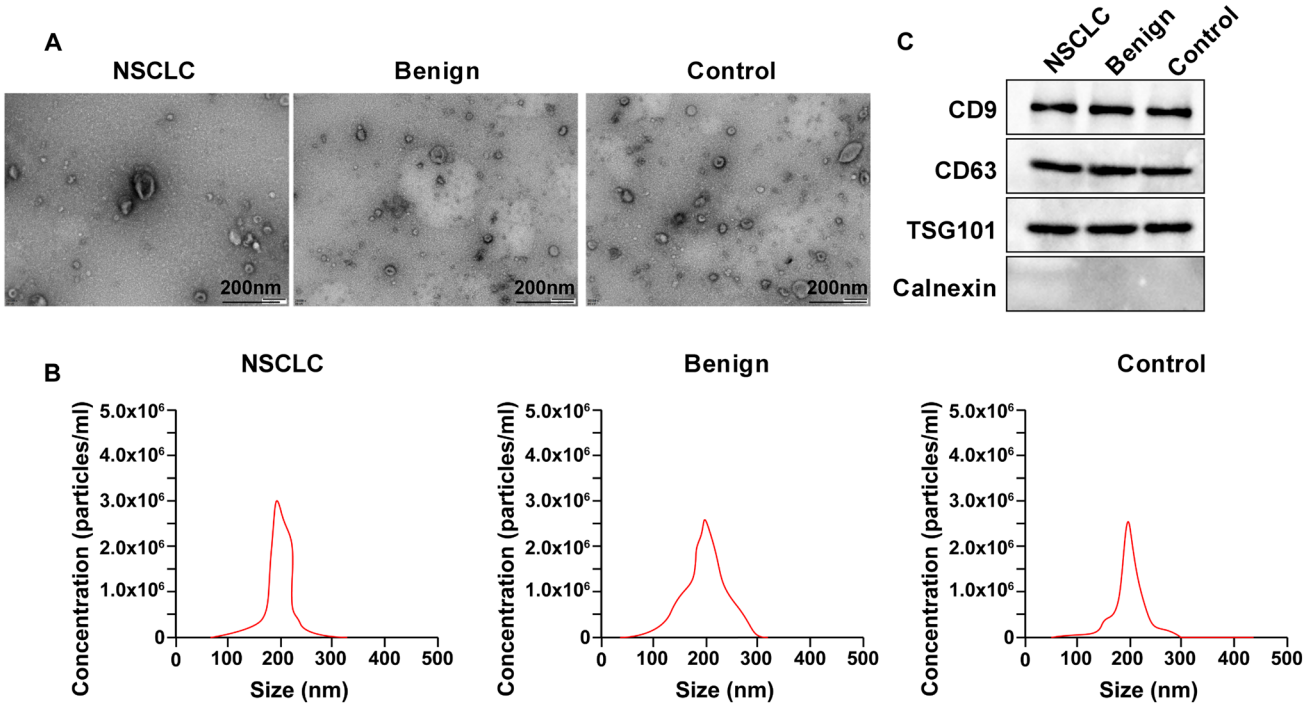

Fig. 1 Identification of peripheral blood-derived EVs. **A** Morphology and structure of EVs observed under the transmission electron microscope. **B** Nanoparticle tracking analysis of the major particle size distribution of EVs. **C** Western blotting to detect the protein expression of EV positive markers CD9, CD63, TSG101 and negative marker Calnexin

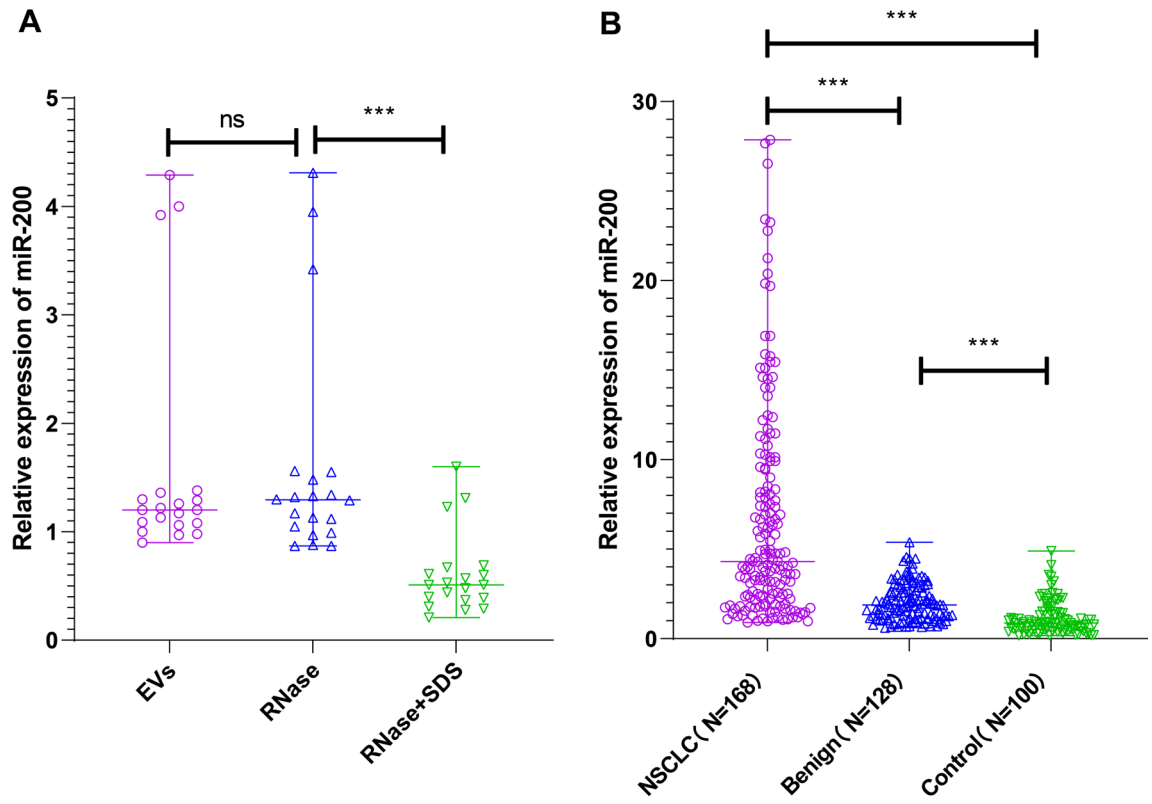

Fig. 2 miR-200 upregulation in peripheral blood-derived EVs of patients with early-stage NSCLC. **A** Detection of miR-200 expression in EVs with different treatments by RT-qPCR. **B** miR-200 expression in peripheral blood-derived EVs of the NSCLC (n = 168), benign (n = 128), and control (n = 100) groups. The Kolmogorov–Smirnov test was performed to analyze the normal distribution of data. Skewed measurement data were presented as quartiles, that is, median (minimum, maximum), and compared among multiple groups with the Kruskal–Wallis rank sum test followed by Tukey's post hoc multiple comparisons test. ***, $p < 0.001$; ns, $p > 0.05$

groups regarding age, sex, FEV1, MVV, FVC, IL-6 and TNF-α ($p > 0.05$). The high miR-200 expression group had higher percentages of patients with a history of smoking and patients with mixed lesions, and higher expression levels of CA242, CEA, and CA199 than the low miR-200 expression group ($p < 0.05$).

Correlation between miR-200 expression in peripheral blood-derived EVs and tumor markers in patients with lung diseases

Further, the Spearman's correlation coefficient was adopted to assess the relationship between miR-200 expression in peripheral blood-derived EVs and tumor markers (CA242, CEA, and CA199) in patients with lung diseases. As observed in Fig. 3, miR-200 expression in peripheral blood-derived EVs from patients with lung diseases was significantly and positively correlated with CA242 (r = 0.901, 95% confidence interval [CI] = 0.8762–0.9209, $p < 0.001$), CEA (r = 0.911, 95%CI = 0.8880–0.9286, $p < 0.001$), CA199 (r = 0.620, 95%CI = 0.5421–0.6873, $p < 0.001$).

Peripheral blood-derived EV miR-200 combined with CA242, CEA and CA199 had high diagnostic value for early-stage NSCLC

The diagnostic value of miR-200 in peripheral blood-derived EVs for early-stage NSCLC was evaluated with a ROC curve, which showed the high diagnostic value of miR-200 in peripheral blood-derived EVs (AUC = 0.855, $p < 0.001$) for early-stage NSCLC, with a high specificity (Fig. 4). Thereafter, the diagnosis of early NSCLC was conducted by combined detection of miR-200 in peripheral blood-derived EVs with CA242, CEA and CA199, with the results indicating the combined detection harbored higher diagnostic value (AUC = 0.942) than them alone, along with a higher specificity (Fig. 4, Table 3). These outcomes suggested that miR-200 in peripheral blood-derived EVs obviously aided in diagnosing early NSCLC.

Table 2 Relationship between miR-200 expression in peripheral blood-derived EVs and clinical characteristics in patients with lung diseases

	High miR-200 expression group (n = 148)	Low miR-200 expression group (n = 148)	*p*
Age (n, %)			
> 50 years	72 (48.65)	67 (45.27)	0.641
≤ 50 years	76 (51.35)	81 (54.73)	
Sex (n, %)			
Male	94 (63.51)	90 (60.81)	0.719
Female	54 (36.49)	58 (39.19)	
Smoking history (n, %)			
Yes	117 (79.05)	98 (66.22)	0.019
No	31 (20.95)	50 (33.78)	
Pathological type (n, %)			
Epithelial type	29 (19.59)	52 (35.14)	< 0.001
Mesenchymal type	34 (22.97)	58 (39.19)	
Mixed type	85 (57.43)	38 (25.68)	
CA242 (U/mL)	28.25 (4.36, 45.96)	17.97 (8.02, 26.74)	< 0.001
CEA (ng/mL)	5.60 (3.48, 13.29)	4.05 (0.36, 4.93)	< 0.001
CA199 (U/mL)	31.56 (9.57, 51.72)	26.18 (17.31, 40.64)	< 0.001
FEV1 (%)	84.49 ± 3.37	85.35 ± 4.66	0.072
MVV (%)	88.07 (76.55, 99.79)	88.95 (69.65, 99.32)	0.153
FVC (%)	80.55 ± 5.46	81.50 ± 6.02	0.157
IL-6 (pg/ml)	67.20 (30.95, 275.74)	68.68 (36.77, 428.39)	0.847
TNF-α (pg/ml)	97.02 (54.50, 256.05)	95.07 (31.72, 452.11)	0.146

The Kolmogorov–Smirnov test was performed to analyze the normal distribution of data. Normally distributed measurement data were displayed as mean ± standard deviation and compared between two groups with the *t*-test. Skewed measurement data were presented as quartiles, that is, median (minimum, maximum) and compared between two groups with the nonparametric test. Intergroup comparisons of categorical variables were analyzed with the Fisher's exact test

CA242 carbohydrate antigen 242, *CEA* carcinoembryonic antigen, *CA199* carbohydrate antigen 19-9, *FEV1* forced expiratory volume in one second, *MVV* maximal voluntary ventilation, *FVC* forced vital capacity, *IL-6* interleukin-6, *TNF-α* tumor necrosis factor-α

High expression of miR-200 in peripheral blood-derived EVs as an independent risk factor for the development of early-stage NSCLC

Subsequently, the indicators with ^{a}p and $^{b}p < 0.05$ in Table 1 were included in the univariate analysis, and the risk factors for the development of early-stage NSCLC were analyzed with univariate and multivariate logistic regression. The results (Table 4) exhibited that age, smoking history, CEA, CA199, TNF-α, and high expression of miR-200 in peripheral blood-derived EVs were the independent risk factors for the development of early-stage NSCLC.

Discussion

Circulating EV miRs are promising non-invasive biomarkers for cancers, which can be utilized for screening, early detection, diagnosis, and prognosis and treatment prediction of cancers [29, 35]. Accordingly, this study screened a reliable peripheral blood-derived EV miR as a marker for the auxiliary diagnosis and disease assessment of NSCLC. In this study, EVs were obtained from peripheral blood by ultracentrifugation and identified with TEM, western blotting, and NTA. Then, a series of experiments showed that miR-200 expression was high in peripheral blood-derived EVs of patients with early-stage NSCLC and that high expression of peripheral blood-derived EV miR-200 was an independent risk factor for early-stage NSCLC and had a high adjunctive diagnostic value for early-stage NSCLC.

The implication of miR-200 in NSCLC has been broadly evidenced [23]. A prior study reported that miR-200 was highly expressed in early-stage lung adenocarcinoma and that miR-200 upregulation fostered cell growth in lung adenocarcinoma [7]. Likewise, another study uncovered that exosomal miR-200 expression was elevated in pleural effusions from patients with lung adenocarcinoma [10]. Moreover, miRs are abundantly expressed in exosomes from plasma and serum [5]. Similarly, our qRT-PCR results displayed that miR-200 in peripheral blood-derived EVs was higher in patients with early-stage NSCLC (4.31 [0.90, 27.86]) than in patients with benign lung nodules (1.89 [0.61,5.39]) and healthy volunteer (0.87 [0.17,4.89]), and treatment with RNase and RNase + SDS Lysis Buffer identified that miR-200 was encapsulated in EVs. The research by Zhong et al. indicated circulating EV miRs as non-invasive detection biomarkers for early-stage NSCLC [35]. Additionally, the research by Zhang et al. revealed that serum exosomal miR-20b-5p and miR-3187-5p were efficient diagnostic biomarkers for early-stage NSCLC [33]. Therefore, peripheral blood-derived EV miR-200 is of high research value in the context of early-stage NSCLC.

CA242 is a sialic acid-containing carbohydrate antigen that is attached to core proteins/lipids, which can be detected on the cell surface or in serum [4]. CA199, a mucin protein, is detected on the glycolipids of cell membranes [36]. CEA, a glycosylated cell surface oncofetal protein, participates in cell adhesion, proliferation, and migration and is abundantly expressed in many cancers [1]. Increased serum levels of CA242, CA199, and CEA have been extensively used as diagnostic biomarkers for cancers in the clinic [4, 11, 14]. In lung cancer, the positive rates of CA199, CEA, and CA242 in LC patients were notably higher than those in

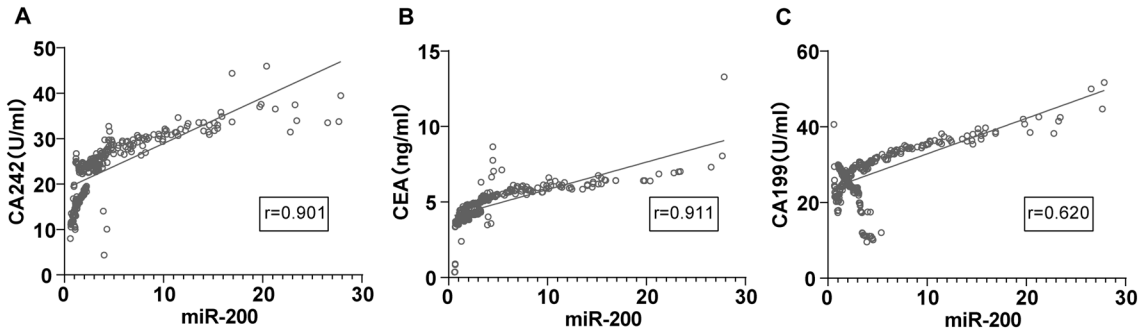

Fig. 3 Relationship between miR-200 expression in peripheral blood-derived EVs and tumor markers in patients with lung diseases. **A**–**C** Spearman's correlation coefficient to analyze the correlation of miR-200 expression in peripheral blood-derived EVs with CA242 (**A**), CEA (**B**), and CA199 (**C**) in patients with lung diseases

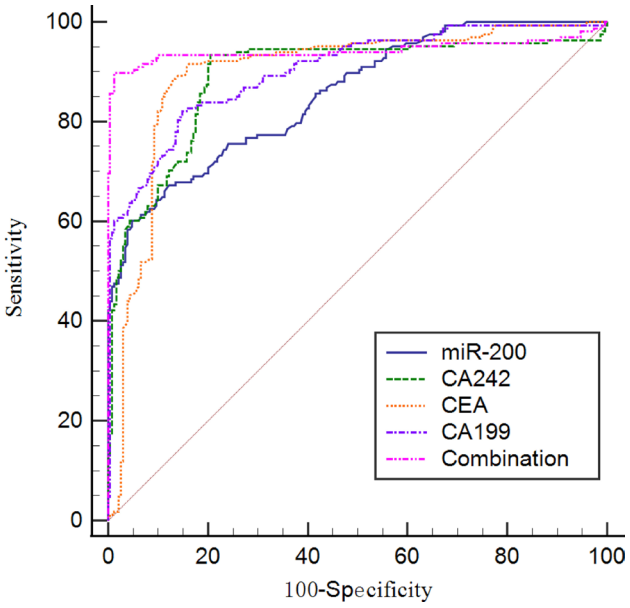

Fig. 4 Diagnosis of early NSCLC. ROC curve was used to analyze the diagnostic value of miR-200 in peripheral blood-derived EVs, CA242, CEA and CA199 for early NSCLC

patients with benign lung disease and healthy people [30]. In the present study, the correlation of peripheral blood-derived EV miR-200 with these three serum tumor markers were analyzed with the Spearman's correlation coefficient to assess the value of peripheral blood-derived EV miR-200 in the diagnosis of lung diseases. The results unraveled that miR-200 expression in peripheral blood-derived EVs from patients with lung diseases was significantly and positively correlated with the levels of CA242, CEA, and CA199.

Diagnostic performance of miR-200 in cancers has been widely investigated. For instance, the diagnostic value of circulating miR-200c in gastric cancer is relatively high, with AUC, sensitivity, specificity, and accuracy rate of 0.715, 65.4%, 100%, and 73.1%, respectively [27]. miR-200s expression in metastatic breast cancer has sensitivity, specificity, and AUC of 0.70 (95%CI = 0.56 − 0.81), 0.72 (95%CI = 0.61 − 0.81), and 0.814 (95%CI = 0.741 − 0.903), respectively, underscoring its high diagnostic accuracy for metastatic breast cancer (Thi Chung [26]). Furthermore, a prior study elucidated that the AUC of peripheral blood-derived exosomal miR-200a-3p, miR-200b-3p, and miR-200c-3p for the diagnosis of cholangiocarcinoma (all > 0.8) was higher that of CA199 (0.78) [25]. However, the diagnostic value of miR-200, particularly peripheral

Table 3 Diagnosis of early NSCLC

Index	Sensitivity	Specificity	AUC	*p*	95%*CI*
miR-200	60.12%	95.18%	0.855	< 0.001	0.816–0.888
CA242	93.45%	79.39%	0.890	< 0.001	0.855–0.919
CEA	88.69%	86.64%	0.898	< 0.001	0.863–0.926
CA199	82.14%	85.09%	0.902	< 0.001	0.868–0.929
Combination	89.88%	98.68%	0.943	< 0.001	0.914–0.964
miR-200 ~ Combination	*P* < 0.0001				
CA242 ~ Combination	*P* < 0.0001				
CEA ~ Combination	*P* = 0.0029				
CA199 ~ Combination	*P* = 0.0006				

Multiple AUC comparisons were performed utilizing the Delong test in the MedCalc software

Table 4 Risk factors for the development of early-stage NSCLC

	Multivariate logistics regression analysis		
	p	OR	95%CI
Age	< 0.001	1.122	1.059–1.188
Smoking history	0.034	2.732	1.081–6.905
CA242	0.253	1.078	0.948–1.227
CEA	0.001	3.160	1.599–6.243
CA199	< 0.001	1.442	1.279–1.625
IL-6	0.250	1.006	0.966–1.016
TNF-α	< 0.001	1.033	1.022–1.044
miR-200	0.020	2.328	1.143–4.744

blood-derived EV miR-200, has been rarely assessed in the domain of LC. Therefore, the current study further evaluated the diagnostic value of peripheral blood-derived EV miR-200 for early-stage NSCLC with the use of a ROC curve, which exhibited that for the diagnosis of early-stage NSCLC, miR-200 in peripheral blood-derived EVs had an AUC of 0.855, and its combination with CA242, CEA and CA199 had an AUC of 0.942, indicating its assistant diagnostic value. Subsequently, it was clarified through the logistic regression analysis that high expression of peripheral blood-derived EV miR-200 was an independent risk factor for the development of early-stage NSCLC.

In summary, this study provided the first survey of the auxiliary diagnostic value of miR-200 in peripheral blood-derived EVs for early-stage NSCLC. Specifically, peripheral blood-derived EV miR-200 expression in patients with lung diseases was closely correlated with the levels of CA242, CEA, and CA199. Moreover, high expression of peripheral blood-derived EV miR-200 was an independent risk factor for early-stage NSCLC and had a high clinical value for the auxiliary diagnosis of early-stage NSCLC. Nevertheless, this study had several limitations. This study was a retrospective single-center study, rendering difficulties in excluding the possibility of potential selection bias. In addition, the included patients were not followed up. Furthermore, miR-200 was not a specific diagnostic indicator for NSCLC, and its relevant expression and significance in SCLC were not explored in this study. Therefore, relevant studies involving follow-up of patients are warranted to further expand the application of peripheral blood-derived EV miR-200 for the prognosis prediction of early-stage NSCLC. Finally, the stability and reproducibility of miRs need to be further verified and explored in the actual clinical application. Overall, our findings illustrate that miR-200 in peripheral blood-derived EVs has an important clinical application value for helping predict the occurrence of early-stage NSCLC and provides an effective guidance for the diagnosis, prevention, and clinical management of early-stage NSCLC.

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

Declarations

Conflict of interests The authors have no conflicts of interest to declare.

Ethics approval and consent to participate The study adhered to ethical guidelines and norms and regulations for clinical trials in the *Declaration of Helsinki* and conformed to the Enhancing the QUAlity and Transparency of Health Research network guidelines. Additionally, the study was ratified by the Academic Ethics Committee of Xi'an People's Hospital (Xi'an Fourth Hospital).

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