Serum exosomal microRNAs as potent circulating biomarkers for melanoma

Li Tengda[a,*], Long Shuping[a,*], Gu Mingli[b,*], Guo Jie[b], Liu Yun[b], Zhang Weiwei[b] and Deng Anmei[a]

Exosomes are small homogenous membrane vesicles that derive from the exocytosis process of cells and can contain DNA, microRNAs (miRNAs), and/or proteins. Characterization of the content profile of exosomes may reflect the state of the cells that release them, and this could be predictive of disease. In this study, to explore the potential biomarkers for melanoma, we isolated serous exosomes from 30 patients with melanoma and 30 healthy individuals using the ultracentrifugation method. Five miRNAs were subsequently detected in each sample by quantitative reverse transcription-PCR: *miRNA-532-5p*, *miRNA-106b*, *miRNA-200c*, *miRNA-199a-5p*, and *miRNA-210*. Only the levels of exo-*miRNA-532-5p* and exo-*miRNA-106b* differed between the two groups ($Z = -4.17$ and -4.57, respectively, $P < 0.0001$). When these two miRNAs were evaluated individually and in combination in 95 melanoma patients and 95 healthy individuals serum samples, the area under the receiver operating characteristic curve values were 0.867, 0.820, and 0.936, respectively. Furthermore, in blinded tests of samples from 25 melanoma patients and 25 healthy individuals, this panel of miRNAs identified 23/25 patients with melanoma (92.0% sensitivity) and 22/25 healthy individuals (88.0% sensitivity). Our exo-miRNA panel also distinguished patients with metastasis from those without metastasis, patients with stage I–II disease from those with stage III–IV disease, and patients who had received pembrolizumab treatment from those who were untreated. Overall, these results indicate that serum exosomal miRNAs, especially exo-*miRNA-532-5p* and exo-*miRNA-106b*, have the potential to be used for monitoring and/or a diagnosis of melanoma in a clinical setting. *Melanoma Res* 00:000–000

Melanoma Research 2018, **00**:000–000

Keywords: exosome, melanoma, microRNA, receiver operating characteristic curve

[a]Center of Clinical Experiments and [b]Department of Laboratory Diagnosis, Changhai Hospital, Second Military Medical University, Shanghai, China

Correspondence to Deng Anmei, PhD, Center of Clinical Experiments, Changhai Hospital, Second Military Medical University, Shanghai 200433, China
Tel: +86 137 6193 9993; e-mail: amdeng70@163.com

or

Correspondence to Li Tengda, MD, Center of Clinical Experiments, Changhai Hospital, Second Military Medical University, Shanghai 200433, China
Tel: +86 187 2197 6819; e-mail: tengdali@smmu.edu.cn

*Li Tengda, Gu Mingli, and Long Shuping contributed equally to the writing of this article.

Received 5 December 2017 **Accepted** 15 March 2018

Introduction

Melanoma is an aggressive tumor that is caused by the malignant transformation of normal melanocytes that produce the pigment, melanin [1]. The original location of a melanoma can vary. According to the National Cancer Database, up to 91% of melanomas are cutaneous melanomas, whereas melanomas located in the eye or in mucosa account for 5.3 and 1.3% of melanoma cases, respectively [2]. Pembrolizumab is a humanized commercial antibody that blocks programmed death (PD)-1 protein binding by its ligand PD-L1 [1]. Treatments blocking the PD-L1/PD-1 pathway have shown very hopeful clinical outcomes in terms of response rate and survival [1]. For advanced metastatic melanomas, therapeutic options are more limited.

Classical biomarkers of melanoma include lactate dehydrogenase (LDH), S100B protein, and melanoma-inhibitory activity (MIA). However, these are not sufficiently sensitive or specific to identify patients with melanoma in its early stages [1,3–5]. Therefore, the identification and development of a biomarker profile that can diagnose early-stage melanoma is essential and critical for improving the prognosis of patients and their survival.

MicroRNAs (miRNAs) are noncoding RNA transcripts that vary in length from 20 to 24 nucleotides. Accumulating evidence has shown potential roles for miRNAs in various diseases, either by controlling the expression of a single gene or by concomitantly regulating multiple genes to influence protein expression [6]. Correspondingly, aberrant regulation of miRNAs has been reported for many tumor types, including melanomas [6]. More recently, it has been shown that serous miRNAs may enhance the identification of melanoma

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website, www.melanoma.com.

DOI: 10.1097/CMR.0000000000000450

patients and provide a valuable parameter for clinical diagnosis [1,6]. However, many routinely used protocols for RNA isolation result in RNA that is partially degraded and/or poor yield of RNA, or the small RNAs are lost in the process [7].

Exosomes are small homogenous membrane vesicles with diameters ranging from 40 to 150 nm that derive from the exocytosis process of cells. Exosomes can contain DNA, miRNAs, and/or proteins and are released into the peripheral circulatory system or the extracellular matrix. On their surface, structural proteins such as CD63 are present, and these are cell membrane-derived and necessary for the integrity of exosomes [8]. Exosomes that contain miRNAs can transport them into neighboring cells by membrane fusion or carry them as a vector to distant organs or issues through circular systems in the body [1,6,8,9]. The immunological activities of exosomes affect immunoregulation mechanisms including intercellular communication and immune activation; besides immune cells, cancer cells secrete immunologically active exosomes that can affect both physiological and pathological processes [1,6,8,9]. It is hypothesized that the content profile of exosomes may reflect the state of the cells that release them, and this could provide valuable biological information on the parent cells [8]. Currently, there are many researchers who believe that the miRNA profiles of tumor exosomes correlate with tumor burden or disease risk. This perspective is based on the valuable bioinformation that miRNAs provide, the stability shown by exosomes, and the large amount of miRNAs that are carried by blood and/or lymphatic fluid in the peripheral circulatory system [8,9].

In this study, the aim was to improve our ability to distinguish patients with melanoma from healthy individuals with sufficient sensitivity and specificity by using a panel of exo-miRNAs to assay serous exosomes after their isolation. For this, we selected two exo-miRNAs out of five exo-miRNAs that were previously found to be aberrantly expressed in tissues or in the peripheral system [6,10–14]. An exo-miRNA panel model was subsequently developed and validated with a large number of clinical samples. Finally, the diagnostic efficiency of this panel model for melanoma was examined.

Patients and methods

Patients

A total of 150 patients with melanoma and 150 healthy individuals were selected for the experimental and control groups for this study, respectively. All samples were collected from Changhai Hospital and Changzheng Hospital (Shanghai, China) between February 2013 and July 2017. Diagnosis standard and stage classification for melanoma were established as described previously [3,15]. Detailed information on the present cohort is presented in Table 1. Patients diagnosed with other types of tumors and pregnant women were excluded from this study. Healthy individuals (67 women and 58 men, mean age: 55.97 ± 9.1 years) were examined at approximately the same time as the patients in the melanoma group.

Ethical statement

This study was approved by the medical research ethics committee of Second Military Medicine University, and the experiments were conducted according to the requirements of the declaration of Helsinki. Written informed consent was obtained from all patients before enrollment.

Isolation of serous exosomes by ultracentrifugation and morphological evaluations

Approximately 600 µl of cell-free serum from each sample was thawed and diluted in 10 ml PBS. After filtration (0.2 µm pore filter) and ultracentrifugation at 160 000*g* at 4°C for 6 h, the obtained precipitates were washed with PBS and ultracentrifuged again at 160 000*g* at 4°C for 2 h. This wash process was repeated one more time before the exosomes were washed with cacodylate buffer and fixed in 4% glutaraldehyde (Polysciences Inc., Warrington, Pennsylvania, USA) for 6 h at 4°C. The samples were subsequently dehydrated with alcohol, stained with uranyl acetate, and scanned with transmission electron microscopy (FEI Co., Hillsboro, Oregon, USA).

Nanosight tracking analysis and zeta potential distribution

The exosome samples were diluted to appropriate concentrations and filtered through 0.2 µm filters. Then, 1 ml of each sample was subjected to nanosight tracking analysis (NTA) using a Nanosight NS300 instrument (Malvern Instruments, Malvern, UK). The main parameters for analysis were as follows: a Blue488 laser, a camera level of 13, a detection threshold of 3, temperatures ranging from 22.9 to 23.1°C, and a syringe pump speed of 30. The experiments were repeated five times to obtain a mean number of exosomes with diameters less than 200 nm. Samples for zeta potential distribution were diluted 10-fold in water and were then analyzed using a Zetasizer Nano instrument (ZS90; Malvern Instruments).

Flow cytometry analysis

Exosomes from serum samples were captured with SM3-P100 magnetic beads (Allrun Nano Science & Technology Co., Shanghai, China) coated with anti-CD63 antibodies (Abcam Co., Cambridge, Massachusetts, USA) or goat anti-mouse IgG antibodies (as a control). Briefly, the SM3-P100 beads were washed twice with 10 mmol/l 2-(*N*-morpholino)ethanesulfonic acid (MES) solution/0.05% Tween 20. Then, the beads were mixed with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (5 mg/ml *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride in 10 mmol/l MES solution) and 200 µl *N*-hydroxysuccinimide (5 mg/ml *N*-hydroxysuccinimide in

Table 1 Relative levels of exo-miR-532-5p and exo-miR-106b that were detected for the various subgroups of patients according to the characteristics listed

Patient characteristics	Patient information [*n* (%)]	Exo-*miR-532-5p* (mean ± SD)	Significance (two tailed)	Exo-*miR-106b* (mean ± SD)	Significance (two tailed)
Age (mean ± SD) (years)	56.01 ± 8.3	–	–	–	–
Sex (female/male)	83/67	–	–	–	–
Tumor stages					
I	29 (19.3)	3.95 ± 2.77	< 0.0001	3.61 ± 2.42	< 0.0001
II	30 (20.0)	4.26 ± 2.39		3.40 ± 2.49	
III	46 (30.7)	5.83 ± 3.54		4.55 ± 2.68	
IV	45 (30.0)	7.14 ± 2.87		6.11 ± 2.81	
Metastasis site					
Lung	22 (14.7)	7.38 ± 3.05	0.269	5.70 ± 3.03	0.526
Liver	19 (12.7)	6.89 ± 2.80		5.07 ± 2.67	
Brain	17 (11.3)	8.08 ± 3.13		4.49 ± 4.14	
Bone	9 (6.0)	5.16 ± 3.14		6.68 ± 3.23	
Other	7 (4.7)	5.57 ± 3.19		5.40 ± 1.57	
Tumor thickness (mm)					
< 1.0	60 (40.0)	4.20 ± 2.61	< 0.0001	3.62 ± 2.54	0.001
≥ 1.0	90 (60.0)	6.44 ± 3.29		5.26 ± 2.82	
Ulceration					
−	76 (50.7)	5.03 ± 3.11	0.051	4.04 ± 2.62	0.021
+	74 (49.3)	6.12 ± 3.27		5.24 ± 2.92	
Primary melanoma					
Cutaneous	81 (54.0)	5.05 ± 3.05	0.026	4.17 ± 2.79	0.007
Mucosal	34 (22.7)	7.00 ± 3.01		6.06 ± 2.60	
Other	35 (23.3)	5.38 ± 3.50		4.28 ± 2.73	

10 mmol/l MES solution), and incubated at 37°C (all of the chemical agents were purchased from Sigma-Aldrich, Burlington, Massachusetts, USA). Bead activation was achieved after 30 min and then appropriate volumes (contained about 50 μg antibodies) of anti-CD63 solution were added to coat the beads. The diluted serum samples were filtered with a 0.22 filter and were then added to the coated beads. The mixtures were rotated at 37°C for 30 min before being separated in a magnetic field. The separated exosomes were incubated with 30 μmol/l Dio reagent (Beyotime Biotechnology Co., Shanghai, China) in ethanol for 30 min and were then resuspended in PBS to an appropriate concentration for flow cytometry analysis (Beckman Coulter, Brea, California, USA) at an excitation wavelength of 484 nm and an emission wavelength of 501 nm. Data were analyzed using Flow Jo 7.6 software (FlowJo LLC, Ashland, Oregon, USA).

RNA isolation and quantitative reverse transcription-PCR

Total RNA was isolated using RNA isolation kits (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. The purity and concentration of the RNA samples were determined using a Nanodrop 2000 instrument (ThermoFisher, Waltham, Massachusetts, USA). If the optical density A260/A280 ratio was greater than 1.8, the samples were analyzed. An M-MuLV One-step RT-PCR Kit (Sangon Biotech, Shanghai, China) was used to detect the relative expression levels of the targeted genes. All of the primers that were used are listed in Supplementary Table S1 (Supplemental digital content 1, *http://links.lww.com/MR/A38*). U6 was detected as the control gene in these experiments. The relative expression of each miRNA was calculated using the $2^{-\Delta\Delta C_t}$ method.

Examination of MIA, LDH, and S100B

MIA was determined using a quantitative enzyme-linked immunosorbent assay kit (Roche, Stuttgart, Germany). Total LDH activity was measured using an automated controlled system (Roche, Stuttgart, Germany). S100B was tested using an Elecsys S100 assay (Roche, Stuttgart, Germany). All of these assays were performed according to the manufacturers' protocols.

Statistical analysis

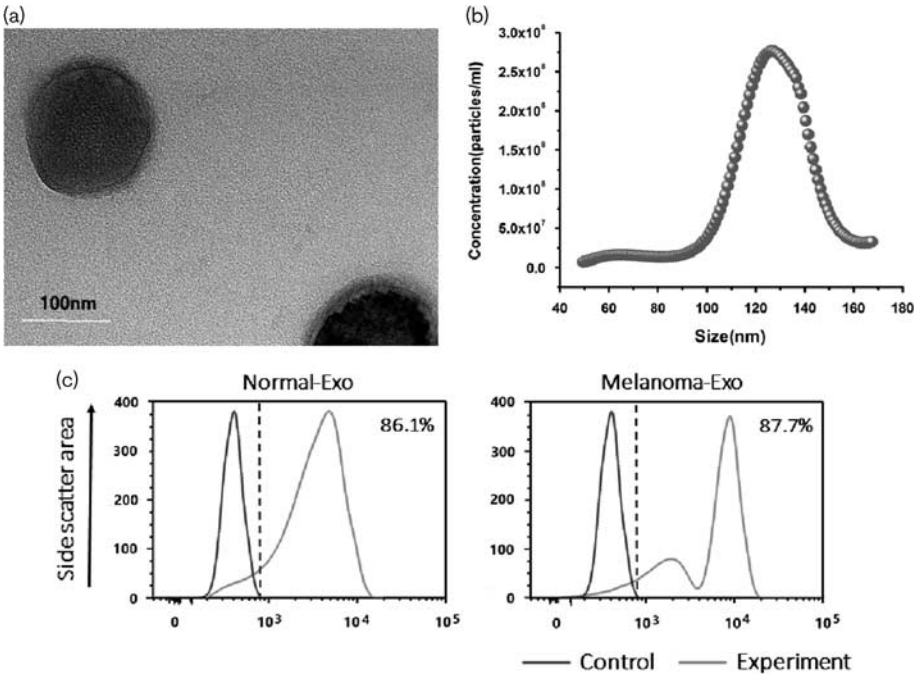
Measurements between two groups were compared using the Mann–Whitney test and measurements between three or more groups were analyzed using the Kruskal–Wallis test. The correlation of two continuous variables was calculated according to Pearson's correlation analysis. Logistic regression was used to develop a combined miRNA panel to predict the possibility of positive results as described previously [16]. All statistical analyses were carried out using IBM SPSS Statistics 21.0 software (IBM company, Palo Alto, California, USA) with a test level of 0.05. Graphs were generated using Graph Prism 6.0 (GraphPad Software, San Diego, California, USA).

Results

Exosome characteristics

Transmission electron microscopy showed that the diameter of the isolated exosomes ranged from 100 to 150 nm (Fig. 1a), whereas NTA estimated the diameter distribution of the exosomes to be 50–170 nm (Fig. 1b). Both sets of data were in accordance with previous estimates [17]. In addition, nanoparticle calculations on the basis of the NTA experiments determined the concentration of exosomes in solution to be 1.05×10^{10}

Fig. 1



Eexosome characteristics. (a) Transmission electron microscopy image of individual exosomes. Scale bar: 100 nm. (b) Graph of the nanosight tracking analysis data. (c) Flow cytometry histograms of CD63 expression by the exosomes (melanoma patients vs. healthy individuals).

particles/ml. Flow cytometry analysis of the exosomes isolated by immunomagnetic beads showed that the exosomes obtained from either normal or melanoma samples showed high levels of expression of CD63, a structural protein that helps exosomes survive [8] (86.1 vs. 87.7%, respectively) (Fig. 1c). The zeta distributions of the serous exosomes obtained from a melanoma patient and a healthy control were − 13.2 and − 22.1 mV, respectively (Supplementary Fig. 1S, Supplemental digital content 1, *http://links.lww.com/MR/A38*). This difference is potentially because of differences in the molecular composition of these two sets of exosomes.

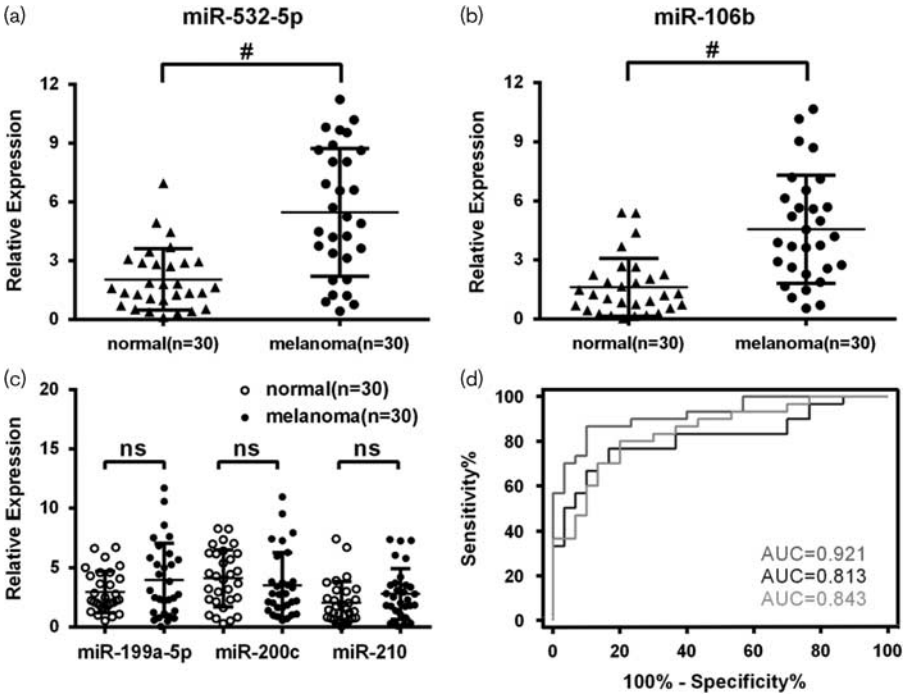
Screening of serous exo-miRNAs and development of a diagnostic panel

Next, we isolated serous exosomes from 30 patients with melanoma and from 30 healthy individuals. Relative expression levels of exo-*miR-532-5p*, exo-*miR-106b*, exo-*miR-199a-5p*, exo-*miR-200c*, and exo-*miR-210* were detected and their distribution profiles are shown in Fig. 2a and c. The Mann–Whitney test was used to compare the relative miRNA levels Supplementary Table S2 (Supplemental digital content 1, *http://links.lww.com/MR/A38*). Only exo-*miR-532-5p* and exo-*miR-106b* were present at significantly higher levels in the serous exosomes collected from the patients with melanoma compared with the exosomes collected from healthy individuals ($P < 0.0001$). We evaluated the diagnostic efficiency of exo-*miR-532-5p* and exo-*miR-106b* for melanoma with receiver operating characteristic (ROC) curves, and the area under the ROC curve (AUC) values were 0.813 [95% confidence interval (CI): 0.701–0.926] and 0.843 (95% CI: 0.745–0.942), respectively. The AUC value of the combined ROC curve of the two miRNAs was 0.921 (95% CI: 0.854–0.989, $P < 0.0001$) (Fig. 2d). Taken together, these data suggest that exo-*miR-532-5p* and exo-*miR-106b* can distinguish melanoma patients from healthy individuals, and their ability to distinguish melanoma is enhanced when they are analyzed in combination.

Validation and blinded test of an exo-miRNA panel

Expression levels of exo-*miR-532-5p* and exo-*miR-106b* were further detected in 95 patients with melanoma and in 95 healthy individuals (Fig. 3a and b). Both exo-miRNAs were present at significantly higher levels in the serous exosomes that were obtained from the patients with melanoma compared with the serous exosomes that were collected from healthy individuals ($Z = -8.73$ and -7.62, respectively, $P < 0.0001$). Moreover, the AUC values of exo-*miR-532-5p* and exo-*miR-106b* for these samples were 0.867 (95% CI: 0.816–0.918) and 0.820 (95% CI: 0.760–0.880), respectively. The combined AUC value was 0.936 (95% CI: 0.903–0.969, $P < 0.0001$) (Fig. 3c). These data imply that the detection of exo-miR-*532-5p* and exo-*miR-106b* can efficiently

Fig. 2



Screening of serous exo-miRNAs expression signature for melanoma diagnosis. (a–c) Relative expression levels of exo-*miR-532-5p* (a); exo-*miR-106b* (b); exo-*miR-199a-5p*, exo-*miR-200c*, and exo-*miR-210* (c) in serous exosomes (30 melanoma patients vs. 30 healthy individuals). (d) Receiver operating characteristic curves for exo-*miR-532-5p* (0.813), exo-*miR-106b* (0.843), and their combination (0.921). $^{\#}P < 0.0001$. AUC, area under the curve.

distinguish patients with melanoma from healthy individuals.

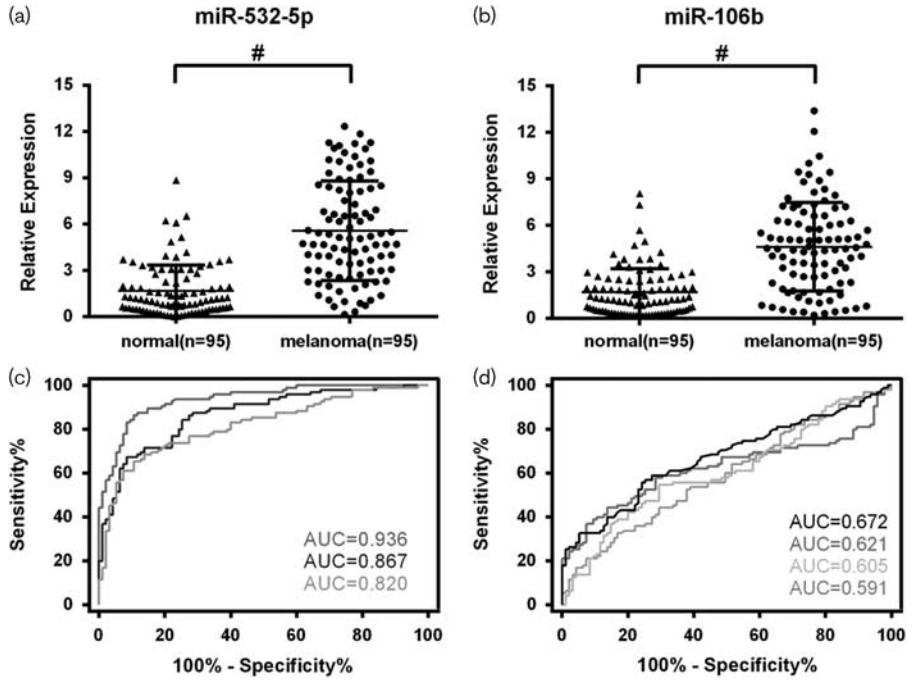
Commonly assessed parameters for melanoma were also examined. Levels of S100B, MIA, and LDH were all elevated in the serum samples obtained from melanoma patients (Table 2), and these results were consistent with those reported previously [4,5]. The AUC values for each were 0.605 (95% CI: 0.524–0.685), 0.591 (95% CI: 0.511–0.672), and 0.621 (95% CI: 0.538–0.704), respectively. Meanwhile, the AUC value for the three assays in combination was only 0.672 (95% CI: 0.595–0.749) (Fig. 3d). All of these AUC values were prominently lower than those for exo-*miR-532-5p*, exo-*miR-106b*, or their combination. Furthermore, a Pearson analysis showed that exo-*miR-532-5p* was related positively to LDH ($r = 0.175$, $P = 0.016$) and MIA ($r = 0.205$, $P = 0.005$), whereas exo-*miR-106b* was related positively to S100B ($r = 0.157$, $P = 0.031$) and MIA ($r = 0.143$, $P = 0.049$) (Table 2). A correlation analysis of exo-*miR-532-5p* and exo-*miR-106b* additionally showed that both were related positively to each other ($r = 0.276$, $P = 0.0001$) (Supplementary Fig. 2S, Supplemental digital content 1, *http://links.lww.com/MR/A38*).

Then, we used the equation below to calculate the combined exo-*miRNAs*: $X = \text{logit}(P) = \ln(P/1-P) = -4.229 + 0.693 \times$ exo-*miR-106b* $+ 0.730 \times$ exo-*miR-532-5p*. For melanoma, predicted probability $(P) = e^X/(1+e^X)$ [16,17]. If the P value was more than 0.63, we considered it positive. When levels of exo-*miR-532-5p* and exo-*miR-106b* were measured in 25 patients with melanoma and 25 healthy individuals whose clinical data were not disclosed, melanoma was predicted in 23/25 of the melanoma patients (92.0% sensitivity) and 22/25 of the healthy patients were confirmed to be free of melanoma (88.0% sensitivity).

Effectiveness of the exo-miRNA panel for detecting various disease conditions

We divided the experimental group into two subgroups: those with and without metastasis and those with stage I–II versus stage III–IV disease (according to the American Joint Committee on Cancer) [3,15]. Detection of serous exo-*miR-532-5p* or serous exo-*miR-106b* could distinguish patients with metastasis from those without ($Z = -4.95$, $P < 0.0001$ and $Z = -2.83$, $P = 0.005$, respectively) and stage I–II patients from stage III–IV patients ($Z = -4.18$ and -3.91, $P < 0.0001$) (Fig. 4), and stage I–II patients from the healthy ($Z = -5.14$ and -3.98, $P < 0.0001$). According to the ROC curves for the former, the AUC values for exo-*miR-532-5p*, exo-*miR-106b*, and their combination for distinguishing patients with and without metastasis were 0.797 (95% CI:

Fig. 3



Validation of serous exo-miRNAs expression signature for melanoma diagnosis. (a, b) Relative expression of exo-*miR-532-5p* (a) and exo-*miR-106b* (b) in serous exosomes (95 melanoma patients vs. 95 healthy individuals). (c) Receiver operating characteristic (ROC) curves for exo-*miR-532-5p* (0.867), exo-*miR-106b* (0.820), and their combination (0.936). (d) ROC curves for lactate dehydrogenase (0.621), S100B (0.605), melanoma-inhibitory activity (0.591), and their combination (0.672). $^{\#}P < 0.0001$. AUC, area under the curve.

Table 2 Levels of LDH, S100B, MIA, and their correlation analysis

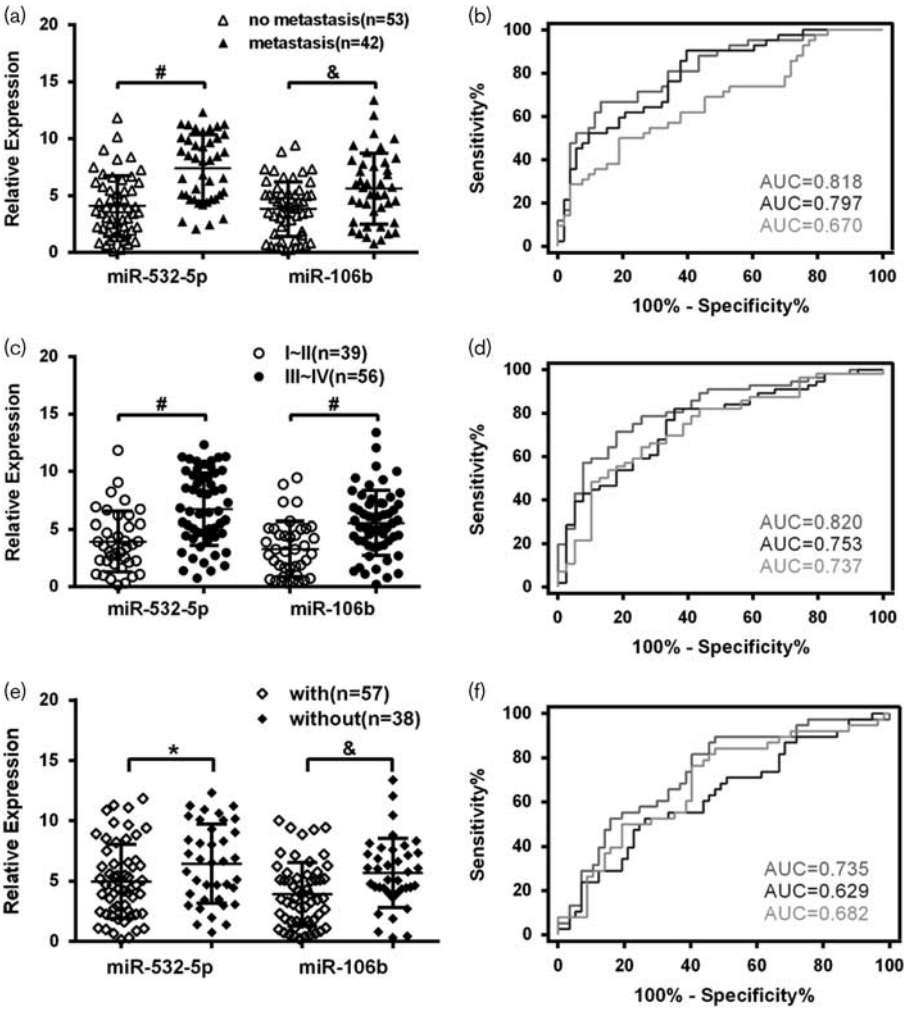
			Exo-miR-532-5p (95 melanoma patients)		Exo-miR-106b (95 melanoma patients)	
Assays	95 melonoma patients vs. 95 healthy individuals	Significance (two tailed)	*r*	Significance (two tailed)	*r*	Significance (two tailed)
LDH (U/l)	199.23 ± 94.99 vs. 162.43 ± 53.72	0.004	0.175	0.016	0.076	0.297
S100B (μg/dl)	0.32 ± 0.23 vs. 0.24 ± 0.19	0.013	0.126	0.082	0.157	0.031
MIA (μg/l)	23.53 ± 20.21 vs. 16.62 ± 13.65	0.030	0.205	0.005	0.143	0.049

LDH, lactate dehydrogenase; MIA, melanoma-inhibitory activity.

0.709–0.885), 0.670 (95% CI: 0.560–0.780), and 0.818 (95% CI: 0.732–0.903, $P < 0.0001$), respectively. The AUC values for identifying stage I–II from stage III–IV for these exo-miRNAs were 0.753 (95% CI: 0.654–0.852), 0.737 (95% CI: 0.634–0.839), and 0.820 (95% CI: 0.734–0.905, $P < 0.0001$), respectively, whereas the AUC values for distinguishing stage I–II from the healthy controls were 0.783 (95% CI: 0.696–0.870), 0.719 (95% CI: 0.620–0.818), 0.836 (95% CI: 0.758–0.915, $P < 0.0001$), respectively (Supplementary Fig. 3S, Supplemental digital content 1, *http://links.lww.com/MR/A38*). We further divided the patients into those who received pembrolizumab treatment and those who did not. The levels of exo-*miR-532-5p* and exo-*miR-106b* were found to differ between these two subgroups ($P = 0.034$ and 0.003, respectively) (Fig. 4e and f), and the AUC values were 0.629 (95% CI: 0.514–0.744) and 0.682 (95% CI: 0.572–0.792). For the combination of both exo-miRNAs, the AUC value was 0.735 (95% CI: 0.633–0.837, $P = 0.0001$).

Finally, we analyzed a total of 150 cases systemically as summarized in Table 1. The Kruskal–Wallis test showed that the relative expression levels of exo-*miR-532-5p* and exo-*miR-106b* differed for stage I–IV patients ($P < 0.0001$), for variable primary melanomas ($P = 0.026$ and 0.007, separately), and they could distinguish tumor thicknesses of less than 1.0 mm versus at least 1.0 mm ($P < 0.0001$ and 0.001, respectively).

Fig. 4



Use of the exo-miRNA panel for distinguishing various disease conditions. Relative expression levels of exo-*miR-532-5p* and exo-*miR-106b* were detected in (a) patients with and without metastasis; (c) patients with stage I–II disease versus stage III–IV disease; and (e) patients receiving or not receiving pembrolizumab treatment. (b, d, f) Receiver operating characteristic curves for exo-*miR-532-5p* (middle), exo-*miR-106b* (bottom), and their combination (top) that were generated for each of the three subgroups evaluated in (a), (c), and (e). $^{*}P < 0.05$, $^{\&}P < 0.01$, $^{\#}P < 0.0001$. AUC, area under the curve.

Discussion

Melanoma is a neoplastic skin disease with a poor prognosis and a high rate of mortality when diagnosed at an advanced stage [2,3]. To date, the therapeutic efficiency for melanoma remains limited, partly because of the late detection of tumors by current diagnostic measures or because of the potential risk of metastasis or subsequent malignant process with histological biopsies [1,18]. With greater attention paid to liquid biopsies, circulating biomarkers represent a noninvasive opportunity for clinicians to obtain dynamic information about the disease state of a patient [8,19]. Unfortunately, serological markers have not been used widely to evaluate whether patients may have developed melanoma because the markers available show low sensitivity or poor specificity [1,5]. For example, LDH was considered to be capable of predicting metastasis risk in uveal melanoma [1]. However, its abnormity only betided a small number of patients with melanoma, even those in late stages. Moreover, serous LDH levels have been shown to increase in response to cellular necrosis [1,4]. Other serous biomarkers, such as S100B or MIA, are also less efficient either because of their short half-life in the peripheral system or their low sensitivity [1,4,5].

To address these problems, greater focus has been placed on investigating circulating miRNAs that disrupt the translation of mRNAs or that destabilize intracellular mRNAs to affect tumor development [11]. Thus, the levels of certain miRNAs in patients with melanoma could potentially provide significant clues on whether a melanoma is present or not [6,11]. It is also important to

note that miRNAs are easily degraded by RNA enzymes that are expressed widely. However, when miRNAs are packaged in exosomes, they are not as easily degraded [11,20]. This result is consistent with the observation that exosomes are vesicles with a very stable bilipidic layer that contains structural proteins (such as CD63) to protect them [21]. Exosomes have been shown to transfer miRNAs from their interior to another targeted location through circulatory systems such as blood [8,21]. On the basis of differences in the proteomic and genomic profiles of tumor cells, it has been proposed that exosomes that are released from tumor cells carry biological information on their parent cells, and this could provide clinical guidance on treatment [8,14,22].

In this study, we found that exo-*miRNA-532-5p* and exo-*miR-106b* showed significant differences in expression levels between 30 patients with melanoma and 30 healthy individuals. Then, we developed an exo-miRNA panel model and validated it with 95 serum samples obtained from both melanoma patients and healthy individuals. The results were encouraging, with a high diagnostic efficiency observed for the two miRNAs. Moreover, the levels detected showed a greater difference than LDH, MIA, or S100B individually or in combination. We have plans to further optimize this exo-miRNA panel model and to evaluate its efficacy for use in routine clinical practice to diagnose melanoma. Part of this goal involves detecting the relative levels of these two exo-miRNAs in each patient and then performing the calculation used in this study: $X = \text{logit}(P) = \ln(P/1-P) = -4.229 + 0.693 \times \text{exo-miR-106b} + 0.730 \times \text{exo-miR-532-5p}$. Then, the predicted probability of melanoma would be calculated as follows: predicted probability $(P) = e^X/(1+e^X)$ [16,17]. With a P value greater than 0.63 (sensitivity: 82.1% and specificity: 91.6%), positivity for melanoma was detected in 23 of 25 melanoma patient samples (sensitivity: 92.0%), and 22 (88.0%) of 25 healthy individuals were not positive for melanoma (data included in Table 1). Furthermore, we found that our exo-miRNA panel could distinguish patients with metastasis from those without metastasis, stage I–II patients from stage III–IV patients, and patients who received and who did not receive pembrolizumab treatment. Taken together, these results suggest that this model could be effective in diagnosing patients with melanoma, and more importantly, could identify patients with early-stage melanoma. The latter is of particular interest to improve the prognosis and quality of life for patients affected by melanoma.

To our knowledge, this study is the first to use a panel of exo-miRNAs to detect patients with melanoma, including early-stage melanoma. Consequently, this panel represents a valuable tool for clinical decisions on treatment. Furthermore, an examination of exosomes in serum is a noninvasive approach that patients may be more amenable to.

Conclusion

Exosomes are vesicles that are released from tumor cells and they can be present in large numbers in blood. Exosomes are generally easy to detect and have been considered an epitome of their parent cells. Thus, the aberrant expressions of exo-miRNAs that were detected in the present article may represent dysregulation that is occurring in the cells from which they were secreted [8,21]. However, the mechanism(s) underlying the observed increases in exo-*miRNA-532-5p* or exo-*miRNA-106b* levels during the process of melanoma development, and/or during an immune response to a tumor, require further study. In addition, the complicated ultracentrifugation method to separate exosomes from plasma or serum samples makes it difficult to use widely in clinical diagnosis. In the future, advanced chemical strategies such as nanochip or immunomagnetic-based technologies which have high sensitivity might be applied to examine exosomes' biological characteristics and form a new approach to achieve high-throughput and more specific detection for clinical biomarkers than the current methods.

Acknowledgements

All the authors thank the patients who were involved in this study. The primers used in this research were from PLA.100 Hospital. Technician Shun-jiang Deng helped us with data management.

This research was supported by grants from the National 973 Foundation (2013CB531606) and the National Science Foundation of China (81671556, 81601406, 81501397, 31500721, 81501398, 81471605, 81401358, 81302579, 81273282, 81202353, and 81701608), and from a Shanghai Shenkang Grant (SHDC22014014), a Shanghai Educational Science Grant (D14017), Army Scientific Research Grants (BWS14J023, 15ZD009,15XD007), and from MJD Founding (MJR20150019).

Conflicts of interest

There are no conflicts of interest.

References

1 Alegre E, Sammamed M, Fernandez-Landazuri S, Zubiri L, Gonzalez A. Circulating biomarkers in malignant melanoma. *Adv Clin Chem* 2015; **69**:47–89.
2 Chang AE, Karnell LH, Menck HR. The National Cancer Data Base report on cutaneous and noncutaneous melanoma: a summary of 84,836 cases from the past decade. The American College of Surgeons Commission on Cancer and the American Cancer Society. *Cancer* 1998; **83**:1664–1678.
3 Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, *et al.* Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol* 2009; **27**:6199–6206.
4 Egberts F, Kotthoff EM, Gerdes S, Egberts JH, Weichenthal M, Hauschild A. Comparative study of YKL-40, S-100B and LDH as monitoring tools for Stage IV melanoma. *Eur J Cancer* 2012; **48**:695–702.
5 Sanmamed MF, Fernandez-Landazuri S, Rodriguez C, Lozano MD, Echeveste JI, Perez Gracia JL, *et al.* Relevance of MIA and S100 serum tumor markers to monitor BRAF inhibitor therapy in metastatic melanoma patients. *Clin Chim Acta* 2014; **429**:168–174.
6 Mirzaei H, Gholamin S, Shahidsales S, Sahebkar A, Jaafari MR, Mirzaei HR, *et al.* MicroRNAs as potential diagnostic and prognostic biomarkers in melanoma. *Eur J Cancer* 2016; **53**:25–32.

7 Cirera S. Highly efficient method for isolation of total RNA from adipose tissue. *BMC Res Notes* 2013; **6**:472.
8 Greening DW, Gopal SK, Xu R, Simpson RJ, Chen W. Exosomes and their roles in immune regulation and cancer. *Semin Cell Dev Biol* 2015; **40**:72–81.
9 Xiao D, Ohlendorf J, Chen Y, Taylor DD, Rai SN, Waigel S, *et al*. Identifying mRNA, microRNA and protein profiles of melanoma exosomes. *PLoS ONE* 2012; **7**:e46874.
10 Lin N, Zhou Y, Lian X, Tu Y. Expression of microRNA-106b and its clinical significance in cutaneous melanoma. *Genet Mol Res* 2015; **14**:16379–16385.
11 Friedman EB, Shang S, de Miera EV-S, Fog JU, Teilum MW, Ma MW, *et al*. Serum microRNAs as biomarkers for recurrence in melanoma. *J Transl Med* 2012; **10**:155.
12 Kitago M, Martinez SR, Nakamura T, Sim M-S, Hoon DSB. Regulation of RUNX3 tumor suppressor gene expression in cutaneous melanoma. *Clin Cancer Res* 2009; **15**:2988–2994.
13 Xu Y, Brenn T, Brown ERS, Doherty V, Melton DW. Differential expression of microRNAs during melanoma progression: miR-200c, miR-205 and miR-211 are downregulated in melanoma and act as tumour suppressors. *Br J Cancer* 2012; **106**:553–561.
14 Fasanaro P, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, *et al*. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. *J Biol Chem* 2008; **283**:15878–15883.
15 Gershenwald JE, Scolyer RA, Hess KR, Sondak VK, Long GV, Ross MI, *et al*. Melanoma staging: evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. *CA Cancer J Clin* 2017; **67**:472–492.
16 Zhang ZN, Xu JJ, Fu YJ, Liu J, Jiang YJ, Cui HL, *et al*. Transcriptomic analysis of peripheral blood mononuclear cells in rapid progressors in early HIV infection identifies a signature closely correlated with disease progression. *Clin Chem* 2013; **59**:1175–1186.
17 Xie Y, Zhang X, Zhao T, Li W, Xiang J. Natural CD8(+)25(+) regulatory T cell-secreted exosomes capable of suppressing cytotoxic T lymphocyte-mediated immunity against B16 melanoma. *Biochem Biophys Res Commun* 2013; **438**:152–155.
18 Aladowicz E, Ferro L, Vitali GC, Venditti E, Fornasari L, Lanfrancone L. Molecular networks in melanoma invasion and metastasis. *Future Oncol* 2013; **9**:713–726.
19 Zhang W, Xia W, Lv Z, Ni C, Xin Y, Yang L. Liquid biopsy for cancer: circulating tumor cells, circulating free DNA or exosomes? *Cell Physiol Biochem* 2017; **41**:755–768.
20 Marta GN, Garicochea B, Carvalho AL, Real JM, Kowalski LP. MicroRNAs, cancer and ionizing radiation: where are we? *Rev Assoc Med Bras* 2015; **61**:275–281.
21 Milane L, Singh A, Mattheolabakis G, Suresh M, Amiji MM. Exosome mediated communication within the tumor microenvironment. *J Control Release* 2015; **219**:278–294.
22 Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, *et al*. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* 2015; **523**:177–182.