Comprehensive serum and tissue microRNA profiling in dedifferentiated liposarcoma

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Abstract. Sarcoma is a rare cancer with several subtypes; therefore, our understanding of the pathogenesis of sarcoma is limited, and designing effective treatments is difficult. Circulating microRNAs (miRNAs), including exosomal miRNAs, have attracted attention as biomarkers in cancer. However, the roles of miRNAs and exosomes in sarcoma remain unclear. The present analysis of tissue and serum miRNA expression in osteosarcoma, Ewing's sarcoma and dedifferentiated liposarcoma (DDLPS) identified miR-1246, -4532, -4454, -619-5p and -6126 as biomarkers for DDLPS. These miRNAs were highly expressed in human DDLPS cell lines and exosomes, suggesting that they are secreted from DDLPS tissues. The present results suggested that specific miRNAs may be used as biomarkers for early diagnosis or treatment targets in DDLPS.

Introduction

Sarcoma has numerous subtypes and typically develops at a relatively young age compared with other cancers (1). Osteosarcoma (OS), the most common primary sarcoma of bone, preferentially develops at 10-14 years of age, which is a period of accelerated bone growth (2,3). Ewing's sarcoma (EWS) is a small round sarcoma and the second most common sarcoma of bone in children (1). Liposarcoma (LPS), a common subtype of soft tissue sarcoma (STS), accounts for 20% of STS cases (4). LPS is classified into 4 categories, which are well differentiated liposarcoma (WDLPS), dedifferentiated liposarcoma (DDLPS), myxoid

liposarcoma and pleomorphic liposarcoma (1,4). DDLPS accounts for ~20% of these and shares a genetic background with WDLPS and occurs focally in the WDLPS lesion (1,5).

microRNAs (miRNAs/miRs) are non-coding RNAs with a length of 17-25 base pairs. miRNAs regulate gene expression at the transcriptional level by binding to target mRNAs (6). Over a period of 10 years of miRNA research, numerous functions of miRNAs have been identified, including their involvement in cancer (7). Exosomes are small vesicles of ~100 nm in size that function as an active transport system in cells (8). Exosomes are enclosed in a lipid bilayer membrane and incorporate mRNAs, miRNAs and proteins (8). Exosomes are released from most cell types and function as intercellular communication tools (8). Cancer cells interact with the microenvironment through exosomes, and tumor-derived exosomes induce premetastatic niches (9).

There are currently no effective biomarkers for sarcomas, to the best of our knowledge. In recent years, circulating miRNAs have attracted attention as candidate biomarkers in several cancers, and these miRNAs are released into the extracellular space by small vesicles, particularly exosomes (10).

Several circulating miRNAs have been identified as candidate markers for OS. The expression levels of miR-195, -Let7A, -9 and -21 are increased in the serum or plasma of patients with OS compared with healthy controls (11-14). miR-135b, -148a, -27a and -9 levels are increased in the exosomes of the serum of patients with OS with a good response to chemotherapy, and these miRNAs can predict the therapeutic efficacy (15).

DDLPS is difficult to detect because it often occurs in the retroperitoneum and trunk (1,16,17). Therefore, it is often discovered after it has grown and is often difficult to resect. When comparing miRNAs in formalin-fixed paraffin-embedded LPS and adipose tissue samples, miR-155 and -21 are upregulated in LPS samples (18,19). High expression of miR-155 and -26a-2 is correlated with a poor prognosis in DDLPS (19,20). miR-25-3p and -92a-3p

are highly expressed in peripheral blood plasma vesicles derived from human LPS patient samples. These miRNAs are secreted from LPS cell lines through extracellular vesicles and transferred to macrophages (21).

In previous work from our group, it was identified that serum miRNAs that can serve as biomarkers for certain types of cancer, such as sarcoma, bladder cancer and ovarian cancer (22-24). However, whether the identified miRNAs are released from the tumor site remains unclear. In the present study, the relevance of the identified miRNAs to sarcoma was determined by comparing miRNA expression levels between serum and tissue samples from the same patient based on the microarray data.

Materials and methods

*Clinical sample*s. All clinical serum samples were obtained from patients undergoing tumor resection at the National Cancer Center Hospital (Tokyo, Japan) (NCCH) between January 2007 and December 2013. The patients that became inoperable during preoperative treatment and were judged to be inappropriate as participants by the doctor or refused to participate in this study were excluded. The clinicopathological data were collected and TNM staging was performed according to French Fédération Nationale des Centres de Lutte Contre Le Cancer (FNCLCC) system described in American Joint Committee on Cancer (AJCC) system, 7th edition (1). Serum samples were stored at 4˚C for 1 week and then stored at -20°C until further use. The serum samples were collected before the operation or preoperative treatment at the NCCH. Tissue samples were obtained from surgical specimens of patients undergoing surgery at the NCCH and stored at -80°C in the NCC Biobank. The study included 22 OS samples, 17 DDLPS samples and three EWS samples (Tables SI-III). All of these samples were used by Asano *et al* (22). The present study was approved by the NCCH Institutional Review Board (Tokyo, Japan; approval nos. 2004-050, 2013-111 and 2015-266). Written informed consent

was obtained from each participant on the first visit. When the patient was under 20 years old, the informed consent was obtained from their parents, relatives or guardians of minors.

miRNA expression array of clinical serum and tissue samples. Serum RNA was extracted from 300 µl of serum using the 3D-Gene® RNA extraction reagent (Toray Industries, Inc.). Fresh-frozen tissues were crushed to a powder using a Multibead Shocker (Yasui Kikai Corporation) under liquid nitrogen (-196˚C). Total RNA was extracted from frozen tumor tissue powder using the miRNeasy Mini kit (cat. no. 217004; Qiagen GmbH). Comprehensive miRNA expression analysis was performed using the 3D-Gene® miRNA Labeling kit and the 3D-Gene® Human miRNA Oligo Chip (both Toray Industries, Inc.), which was designed to detect 2,588 miRNA sequences registered in miRBase release 21 database (http://www.mirbase.org/). Microarray experiments were performed by Kamakura Techno-Science Inc. miRNAs with a signal intensity $>2^6$ were considered detected miRNAs. Principal component analysis (PCA) map and heatmap were generated by Genomics Suite version 6.6 (Partek Inc.).

Cell culture. Human DDLPS cell lines (LP6 and LPS12) were previously established and kindly provided by Dr Andrew J. Wagner (Dana Farber Cancer Institute; USA). Human adipose-derived stem cells (ADSCs) were purchased from Invitrogen; Thermo Fisher Scientific, Inc (cat. R7788115). LP6 cells were cultured in RPMI 1640 medium (cat. 11875093; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; cat. 10270-106; Thermo Fisher Scientific, Inc.). LPS12 cells were cultured in DMEM/F12 (cat. 21331020; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and GlutaMAX (cat. 35050061; Thermo Fisher Scientific, Inc.). ADSCs were cultured in MesenPRO RS™ medium (cat. 12746012; Thermo Fisher Scientific, Inc.). All cell cultures were treated with 1% antibiotic-antimycotic solution (cat. 15240062; Thermo Fisher Scientific, Inc.) and cultured at 37°C in

5% CO_2.

Cell proliferation assay. LP6 cells were seeded at a density of $5x10^3$ cells per well into a 96-well dish at 37°C in 5% CO_2. After 1 day of seeding, the medium was replaced by miRNA mimics (hsa-miR-1246, ID MC13182; 5'AAUGGAUUUUUGGAGCAGG-3'; hsa-miR-4532, ID MC21908; 5'-CCCCGGGGAGCCCGGCG-3'; hsa-miR-4454, ID: MC21186; 5'-GGAUCCGAGUCACGGCACCA-3'; hsa-miR-619-5p, ID: MC28761; 5'-GCUGGGAUUACAGGCAUGAGCC-3'; and hsa-miR-6126, ID: MCMC25200; 5'-GUGAAGGCCCGGCGGAGA-3'; all Thermo Fisher Scientific, Inc.) in DharmaFECT reagent-1 (cat. no. T-2001-02; GE Healthcare Dharmacon, Inc.) for miRNA transfection. Negative Control #1 (NC) (cat. no. 4464058; Thermo Fisher Scientific, Inc.) was used as a non-targeting negative control. The concentrations of miRNA mimics and NC were 2 μM in the original stocks. The transfection efficiency was estimated (n=4) based the average using HiLyte Fluor488 negative control miRNA mimic (Nippon Gene Co., Ltd.) and evaluated using reverse transcription-quantitative PCR with miRNA-specific primers. Each miRNA was added to three wells. For all miRNA mimic transfection, final concentrations were 10 nM. The time of transfection was 8 h at 37˚C in 5% CO_2. After transfection (24, 48 and 72 h), the number of living cells was counted using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) for 3 days, and measurements (Gen5 Synergy H4; BioTek Instruments, Inc.) were performed three times. The average value of day 2 and 3 was normalized to that of day 1. Proliferation curves were drawn with the average value, and the standard deviation was calculated. Images were captured using a fluorescent microscope (BZ-X700; Keyence Corporation).

Purification and analysis of exosomes. Exosomes were prepared as previously described (25). Briefly, to avoid the contamination of exosomes from FBS, cells were washed with PBS and

the culture medium was replaced with advanced RPMI medium (cat. 12633012; Thermo Fisher Scientific, Inc.) with 2 mM L-glutamine (cat. 25030149; Thermo Fisher Scientific, Inc.) for LP6 cells and advanced DMEM/F12 medium (cat. 12491015; Thermo Fisher Scientific, Inc.) with 2 mM L-glutamine for LPS12 cells. The ADSC culture medium was replaced by StemPro™ MSC SFM medium (cat. A1033201; Thermo Fisher Scientific, Inc.). The LP6, LPS12 and ADSC cells were incubated for 48h at 37˚C in 5% CO_2. After 48 h of incubation, the conditioned medium (CM) was collected and centrifuged at 2,000 x g for 10 min at 4°C. The supernatant was filtered through a 0.22-µm filter (EMD Millipore). The filtered CM was ultracentrifuged at 110,000 x g using a SW41Ti rotor for 70 min at 4°C using an Optima XPN-100 Ultracentrifuge (Beckman Coulter, Inc.). The supernatant was discarded, and the pellet was washed by adding PBS , ultracentrifuged at 35,000 rpm 110,000 x g using the SW41Ti rotor for 70 min at 4˚C, and resuspended in PBS. For determination of the size distribution of exosomes, nanoparticle tracking analysis was performed using the NanoSight system (NanoSight; Malvern Panalytical, Ltd.) in samples diluted 500-1,000-fold with PBS for analysis (25).

RNA extraction and RT-qPCR. miRNAs were isolated from the LP6, LPS12 and ADSC cells and exosomes using the miRNeasy® Mini kit (cat. no. 217004; Qiagen GmbH), and cDNA was produced using the miScript II RT kit (cat. no. 218160; Qiagen GmbH) according to the manufacturer's instructions. The cDNA samples with miScript primers were subjected to quantitative PCR using a miScript® SYBR® Green PCR kit (cat. no. 218075; Qiagen GmbH). Reactions were performed three times on the StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following specific primers were used: Hs_miR-1246_1 (cat. no. MS00014224), Hs_miR-4532_1 (cat. no. MS00040705), Hs_miR-4454_1 (cat. no. MS00037597), Hs_miR-619-5p_1 (cat. no. MS00046032) and Hs_miR-

6126_1 (cat. no. MS00045416) (all Qiagen GmbH). The expression levels were normalized to those of RNU6-2_11 (cat. no. MS00033740; Qiagen GmbH), and relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (26). The expression levels of each miRNA in LP6 and LPS12 were compared with those in ADSCs to calculate the fold-changes. The average values and standard deviations were calculated.

Immunoblotting. In total, 1 μg of exosomes were extracted by Sample Buffer Solution (2-Mercaptoethanol -) (FUJIFILM Wako Pure Chemical Corporation) and 1 μg of proteins were loaded per lane onto 4-15% Mini-PROTEAN TGX gels (Bio-Rad Laboratories, Inc.) and electrotransferred (100 V, 30 mA) (27). The proteins were transferred to polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked with Blocking One solution (cat. 03953-95; Nacalai Tesque) on shacking machine for 1 h at room temperature and then incubated for 1 h at room temperature with primary antibodies: anti-CD63 (1:1,000; cat. no.12A12; Cosmo Bio Co., Ltd.) and anti-CD9 (1:1,000; cat. no. 8A12; Cosmo Bio Co., Ltd.). After washing, the membranes were incubated for 1 h at room temperature with secondary antibodies (horseradish peroxidase-linked anti-mouse IgG; cat. no. NA931 or horseradish peroxidase-linked anti-rabbit IgG; cat. no. Na934; both 1:5,000; GE Healthcare). After washing, the membranes were then exposed to ImmunoStar LD for development (cat. 292-69903; FUJIFILM Wako Pure Chemical Corporation).

Statistical analysis. miRNA expression levels were analyzed using Pearson's correlation, with the vertical axis representing tissues and the horizontal axis representing serum. The expression levels of miRNAs in cells and exosomes were compared by calculating the average values and the standard deviation. The data are presented as mean ± SD of 3 or 4 biological replicates. A total of 3 biological replicates were performed for the experiments of miRNA

extraction from cell lines and exosomes and cell proliferation. A total of 4 biological replicates were performed of the transfections of fluorescently-labeled miRNA mimic. The significance of the average values was analyzed using one-way ANOVA with a Tukey's HSD post hoc test. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using Excel 2019, Microsoft and online (One-way ANOVA with post-hoc Tukey HSD Test Calculator for comparing multiple treatments) (https://astatsa.com/OneWay_Anova_with_TukeyHSD/).

Results

Expression of miRNAs in the tissues and serum of patients with sarcoma. A total of 22 OS, 17 DDLPS and three EWS samples were analyzed. Overall, 830 miRNAs were detected in tissues and 142 miRNAs in serum (Fig. 1A), and 141 miRNAs were expressed both in tissues and serum. Fig. 1B shows the results of hierarchical unsupervised clustering with miRNAs on the vertical axis and clinical cases on the horizontal axis. The expression of miRNAs was generally higher in tissues compared with in serum. Based on the PCA map (Fig. 1C), DDLPS and OS samples were separated in both serum and tissue samples. The expression profiles in tissues and serum were separated in the PCA map (Fig. 1C, right panel). Different trends were observed in miRNA expression patterns for each tumor type and miRNAs that were highly expressed in both tumor and serum miRNAs were found.

Correlation coefficients of sarcoma samples and DDLPS samples. The Pearson's correlation coefficients in miRNA expression between the serum and tissues were calculated (Fig. 2A). In the histograms of Fig. 2A, the distribution of Pearson correlation coefficient (R) was shown. In OS and DDLPS, there were a few miRNA which showed positive correlation between the serum and the tissues (Fig. 2A). Although miRNAs in EWS samples were expressed at high

levels, the number of detected miRNAs was too low (n=3) to determine statistical significance. However, several miRNAs in DDLPS were expressed at relatively high levels, including miR-1246, -4532, -4454, -619-5p and -6126. The correlation these miRNAs in tissues and sera in DDLPS is presented in Fig. 2B. The expression levels of certain miRNAs, such as -1246, -4532 and -619-5p, were weakly correlated between serum and tissue samples (R =0.33-0.49), suggesting that these serum miRNAs could be derived from tumors in patients with DDLPS.

miRNA expression in DDLPS cell lines and effect on cell proliferation. It was examined whether the identified miRNAs were released from the human DDLPS cell lines LP6 and LPS12 (Fig. 3A). For measurement of the expression levels of the miRNAs in DDLPS cells, total RNA was extracted from LP6 and LPS12 cells and ADSCs as controls and subjected to quantitative PCR. The expression of miR-1246, -4532, -4454 and -619-5p was higher in LP6 and LPS12 cells compared with in ADSCs (Fig. 3B). The ratio of the expression level of miR-1246 was 3.52±0.89 ($P<0.01$) in LP6 and 2.87±0.52 ($P<0.01$) in LPS12 cells. That of miR-4532 was 1.36±0.43 ($P=0.29$) in LP6 and 2.48±0.28 ($P<0.01$) in LPS12 cells. That of miR-4454 was 1.90±0.47 ($P<0.01$) in LP6 and 3.72±0.52 ($P<0.01$) in LPS12 cells. That of miR-619-5p was 2.36±0.99 ($P<0.01$) in LP6 and 1.88±0.84 ($P=0.02$) in LPS12 cells. That of miR-6126 was 0.62±0.12 ($P<0.01$) in LP6 and 0.83±0.15 ($P=0.03$) in LPS12. To examine the effects of these miRNAs on DDLPS cell proliferation, the transfection efficiency of miRNA in LP6 cells was examined using a fluorescently-labeled miRNA mimic negative control. Based on the fluorescence rate, efficiency was 92.5±3.3% (Fig. 3C). Then, LP6 cells were transiently transfected with miRNA mimics, and the overexpression of each miRNA was confirmed using RT-qPCR (Fig. 3D). The cell proliferation rates were monitored on days 1, 2 and 3 after transfection (Fig. 3E). The cells transfected with the miRNAs exhibited a higher proliferative capacity compared with the negative control. miR-4532 had a significant effect on promoting

cell proliferation in LP6 cells ($P<0.05$). miR-1246, -4454 and -619-5p were significantly highly expressed in both DDLPS cell lines. This result indicated the possibility that these miRNAs are also highly expressed in DDLPS tissues.

DDLPS cells release exosomes containing specific miRNAs. Exosomes were isolated from LP6 cells, LPS12 cells and ADSCs by ultracentrifugation. Isolated exosomes were confirmed using the NanoSight system (Fig. 4A). Typical exosome markers were confirmed with immunoblotting (Fig. 4B). CD63 and CD9 were expressed in the exosomes from LP6, LPS12 and ADSCs. Also, we previously confirmed the isolation of exosomes from ADSC by transmission electron microscopy (28). Total RNA was extracted from exosomes, and the expression levels of miRNAs were examined using RT-qPCR (Fig. 4C). miRNA expression levels were lower in exosomes from LPS12 cells compared with those from LP6 cells. The results of RT-qPCR analysis showed that miR-1246, -4454 and -619-5p were highly expressed in LP6 exosomes. The ratio of the expression level of exosomal miR-1246 was 1.92±0.89 ($P<0.01$) in LP6 and 0.42±0.52 ($P<0.01$) in LPS12 cells. That of miR-4532 was 0.56±1.91 ($P<0.01$) in LP6 and 0.18±0.37 ($P<0.01$) in LPS12 cells. That of miR-4454 was 8.35±0.16 ($P<0.01$) in LP6 and 0.28±0.03 ($P=0.02$) in LPS12 cells. That of miR-619-5p was 3.36±0.58 ($P<0.01$) in LP6 and 0.46±0.04 ($P=0.67$) in LPS12 cells. That of miR-6126 was 0.23±0.88 ($P<0.01$) in LP6 and 0.28±0.02 ($P<0.01$) in LPS12 cells. These results indicated that miR-1246, -4454 and -619-5p detected in the serum were derived from sarcoma tissues. Exosomal miR-1246, -4454 and -619-5p were detected in LP6 cells. This result indicated the possibility that these miRNAs are present in DDLPS serum.

Discussion

The use of multidrug chemotherapy in combination with surgery strongly improves the

outcomes of patients with OS, resulting in a 5-year overall survival rate of ~64-77% globally (29-32). However, the 5-year survival rate of patients with OS with metastatic disease and relapse at the first visit is <20% globally (1,33). The mortality rate of DDLPS is ~60% globally, which can be partly attributed to tumors that are too large to be resected at the time of diagnosis (34). The present study identified miRNAs with potential value as biomarkers for DDLPS. A correlation between the serum and tissue expression of specific miRNAs [miR-1246 (R = 0.49), miR-619-5p (R = 0.33), miR-4532 (R = 0.33), miR-4454 (R = 0.21) and miR-6126 (R = 0.20)] and measured the expression levels of these miRNAs in DDLPS cell lines (LP6 and LPS12) and released exosomes.

Most of the miRNAs showing high levels of expression in the serum were also highly expressed in tissues in the present study, suggesting that serum miRNAs could be derived from tumor tissues. Although the number of EWS samples was too small to draw firm conclusions, the number of OS and DDLPS samples was considered to be sufficient for the analysis. There were differences in the miRNA expression between serum and tissues. When these miRNAs were evaluated individually, miR-1246 showed the highest correlation coefficient. To determine whether miRNAs were secreted from DDLPS cells, the miRNA expression levels between cells and exosomes were compared. miR-1246, -4454 and -619-5p were highly expressed in both DDLPS cell lines. miR-1246, -4454 and -619-5p were highly expressed in LP6 exosomes, even though the expression levels of miRNAs were generally low in LPS12 cells. The different results of these two cell lines may be because the cell line was isolated from part of one sample and may not have the characteristics of the original tumor. These data indicated that miR-1246, -4454 and -619-5p may be candidate miRNAs for the diagnosis of DDLPS. However, these miRNAs were not candidates for other LPS subtypes (well differentiated, myxoid/round cell or pleomorphic liposarcomas).

There are few studies on miRNA biomarkers of sarcomas, but several studies have

reported candidates for OS biomarkers (11-14,35). miR-195-5p, -199a-3p, -320a and -374a-5p were significantly increased in the plasma of patients with OS compared with healthy controls (35). The expression of these four miRNAs decreased after tumor resections which were 83 extremities and 7 trunks and the expression of miR-195-5p and -199a-3p was significantly increased in patients with metastasis (35). miR-9 and -21 are upregulated in the blood samples of patients with OS, but -195, -Let7A, -199a-3p and -143 are downregulated (11-14). These studies differed from the present report in that they compared blood samples of patients with OS with those of healthy controls. Moreover, Fujiwara *et al* (36) identified miR-25-3p as a diagnostic and prognostic biomarker of OS *in vitro*, *in vivo* and in clinical samples.

miR-155 was previously reported as a biomarker for the diagnosis of DDLPS. miR-155 is highly expressed in the tissues and plasma (37). Similarly, the high expression of miR-155 in tissues is correlated with poor prognosis (19). The present study examined the correlation between miRNA expression in the serum and tissue and serum miRNA levels may be used for predicting patient with DDLPS prognosis.

The present assessment of the effect of miRNAs on cell proliferation showed that miR-4532 and -6126 significantly promoted DDLPS cell proliferation. This finding suggested that these miRNAs may promote the progression of DDLPS. Meanwhile, other papers have reported that miR-4532 promotes tumor progression and that miR-6126 suppresses tumor progression. Breast cancer cells overexpressing miR-4532 have enhanced cell viability upon administration of adriamycin (38). miR-4532 targeting hypermethylation in cancer 1 (HIC-1), and overexpression of HIC-1 in breast cancer cells suppresses cell invasion in Transwell assays (38). Conversely, enhanced expression of miR-6126 suppresses ovarian cancer progression. Ovarian cancer cells transfected with miR-6126 mimic have decreased cell migration and invasion (39). The reason for the discrepancy between these previous studies and the present results is the different cancer types. In addition, the present evaluation was insufficient because

only cell proliferation was evaluated. Additional experiments are required to identify direct targets of miR-4532 and miR-6126 in DDLPS.

The present study identified specific miRNAs that were highly expressed in both the serum and tissues from patients with DDLPS, and *in vitro* experiments suggested that certain miRNAs were secreted from DDLPS cells. Taken together, the present results suggested that the identified miRNAs could be of value as biomarkers in DDLPS.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

IK, NA, JM, YY, TY and RT performed the experiments, analyzed data and wrote the manuscript. EK, HC, AK and TO conceived the study, and analyzed and interpreted the data. ST, HS, KK and HF contributed to data analysis. IK, JM and YY confirmed the authenticity of raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by The National Cancer Centre Hospital Institutional Review Board (Tokyo, Japan; approval nos. 2004-050, 2013-111 and 2015-266). The reason of separated approval number was that we collected the data from several studies for this re-analysis. Written informed consent was obtained from each participant. When the patient was under 20 years old, the informed consent was obtained from their parents, relatives or guardians of minors.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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471 Suppressor miR-6126 from Ovarian Cancer Cells. Cancer Res 76: 7194-7207, 2016.
472
473
474 **Figure legends**

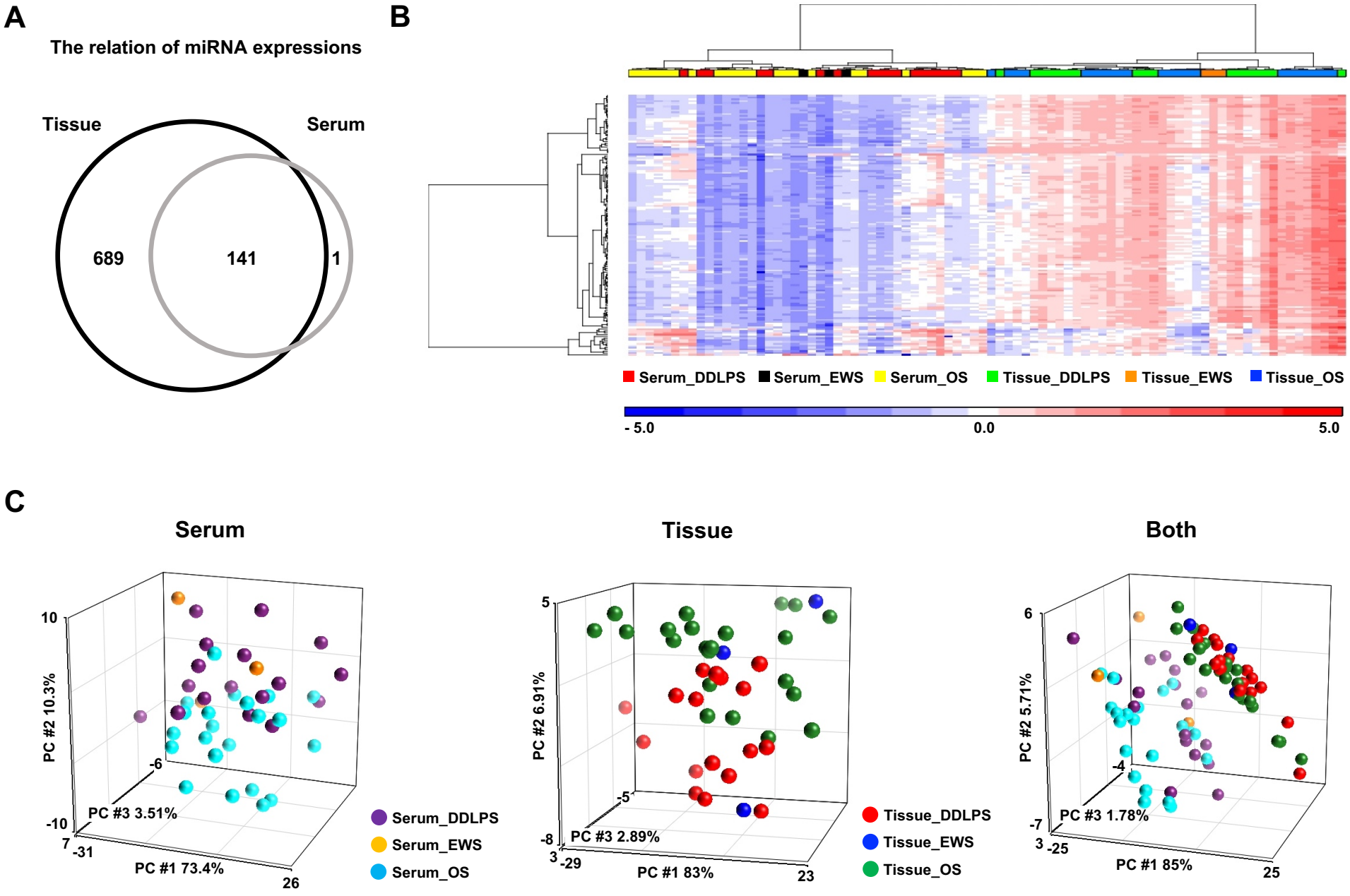
Figure 1. miRNA expression in the tissues and serum of patients with sarcoma. (A) Number of miRNAs with a signal intensity $>2^6$ in tissues and serum. (B) Heatmap of miRNAs in tissue and serum samples (the vertical axis represents miRNAs and the horizontal axis represents the serum and tissue samples for each subtype). (C) Principal component analysis map of each serum and tissue sample. miRNA, microRNA; DDLPS, dedifferentiated liposarcoma; EWS, Ewing's sarcoma; OS, osteosarcoma.

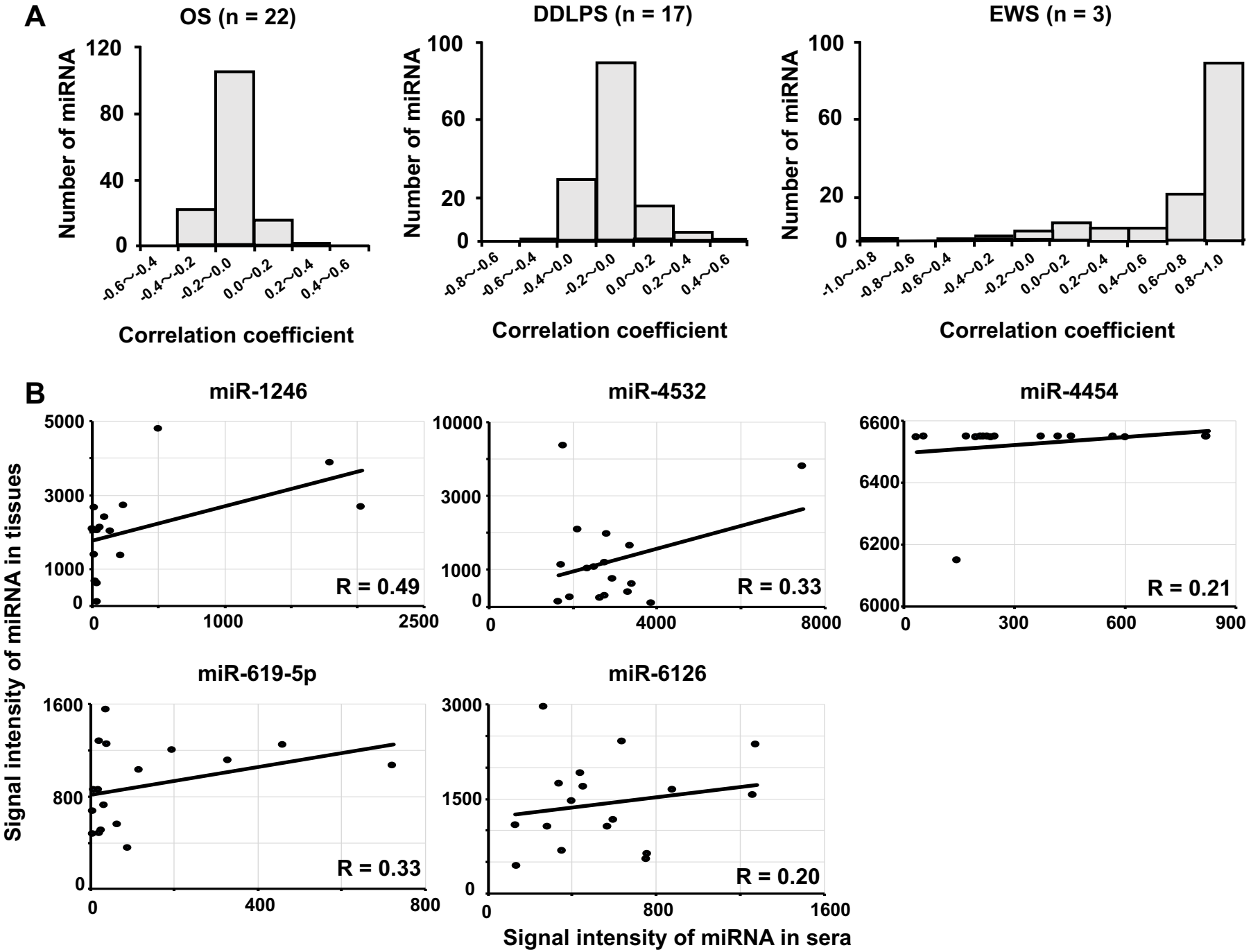
Figure 2. Correlation coefficients of sarcoma samples and regression curves of DDLPS. (A) Histograms of the correlation of the expression levels of miRNAs between tissues and serum (the vertical axis represents the number of miRNAs, and the horizontal axis represents the value of the correlation coefficient). (B) Correlation curves of five miRNAs showing a high correlation coefficient in DDLPS. miRNA/miRs, microRNA; DDLPS, dedifferentiated liposarcoma; EWS, Ewing's sarcoma; OS, osteosarcoma.

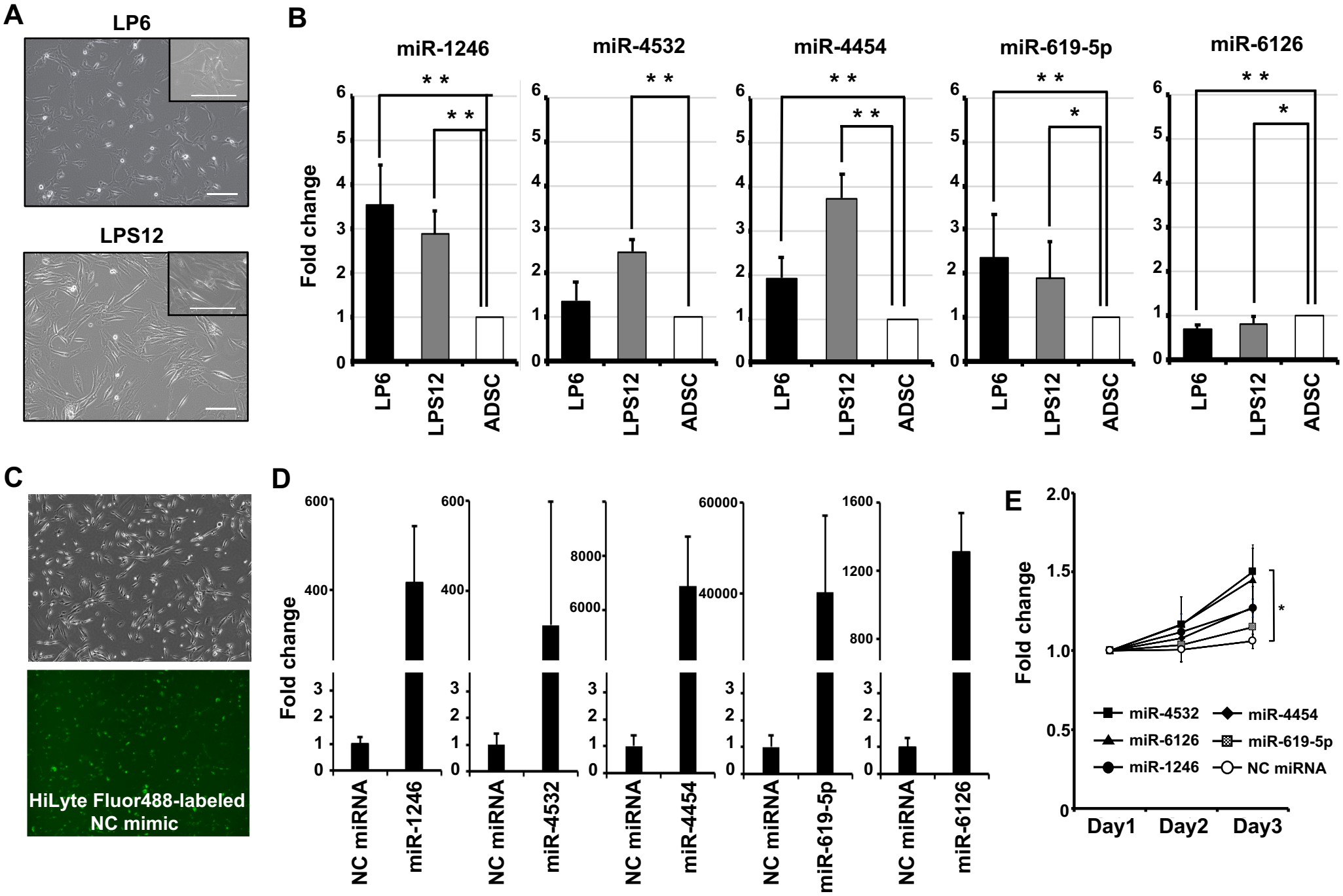
Figure 3. Expression of selected miRNAs in DDLPS cell lines and effect on cell proliferation. (A) Representative images of DDLPS cell lines. Scale bar, 200 μm. (B) Expression levels of each miRNA in LP6 and LPS12 cells normalized to that of ADSCs. (C) Transfection rate estimation using HiLyte Fluor488-labelled NC miRNA mimic in LP6 cells. Upper: phase contrast; lower: fluorescent image. Magnification, x40. (D) Reverse transcription quantitative-PCR analysis of miRNAs after miRNA mimic transfection. Fold-changes was calculated by normalizing miRNA levels to the NC values. The value of negative control set to 1.0. (E) Cell proliferation assay using LP6 cells. Each miRNA was transfected to LP6 cells. $^{*}P<0.05$ and $^{**}P<0.01$. NC, negative control; miRNA/miR, microRNA. ADSC, adipose-derived stem cells.

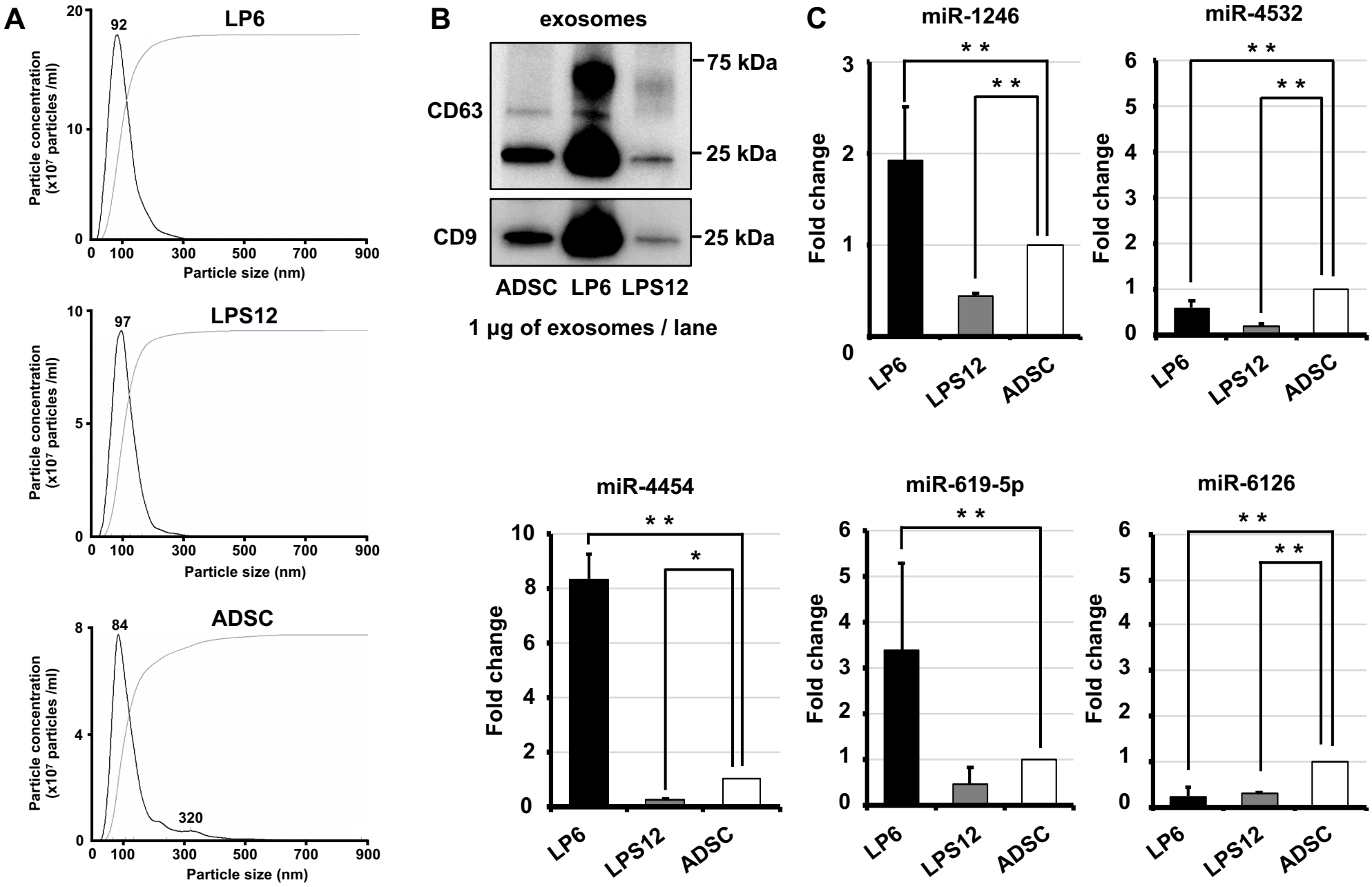
Figure 4. Detection of selected miRNAs in the exosomes of DDLPS cells. (A) Size evaluation

of collected exosomes using the Nano tracking system. Number at the vertex of the graph indicates the particle size. (B) Immunoblotting for CD63 and CD9 in LP6 and LPS12 cells and ADSC. (C) Expression of each miRNA in exosomes from LP6 and LPS12 cells was normalized to that of ADSCs. $^{*}P<0.05$ and $^{**}P<0.01$. miRNA/miR, microRNA; ADSC, adipose-derived stem cells.


A
The relation of miRNA expressions
Tissue
Serum
689
141
1
B
Serum_DDLPS
Serum_EWS
Serum_OS
Tissue_DDLPS
Tissue_EWS
Tissue_OS
- 5.0
0.0
5.0
C
Serum
PC #2 10.3%
PC #3 3.51%
PC #1 73.4%
Serum_DDLPS
Serum_EWS
Serum_OS
Tissue
PC #2 6.91%
PC #3 2.89%
PC #1 83%
Tissue_DDLPS
Tissue_EWS
Tissue_OS
Both
PC #2 5.71%
PC #3 1.78%
PC #1 85%


A
OS (n = 22)
DDLPS (n = 17)
EWS (n = 3)
Number of miRNA
Correlation coefficient
B
miR-1246
miR-4532
miR-4454
miR-619-5p
miR-6126
R = 0.49
R = 0.33
R = 0.21
R = 0.33
R = 0.20
Signal intensity of miRNA in tissues
Signal intensity of miRNA in sera


A
LP6
LPS12
B
miR-1246
miR-4532
miR-4454
miR-619-5p
miR-6126
Fold change
LP6
LPS12
ADSC
C
HiLyte Fluor488-labeled
NC mimic
D
Fold change
NC miRNA
miR-1246
miR-4532
miR-4454
miR-619-5p
miR-6126
E
Fold change
miR-4532
miR-4454
miR-6126
miR-619-5p
miR-1246
NC miRNA
Day1
Day2
Day3


A
LP6
92
Particle concentration (x10⁷ particles /ml)
Particle size (nm)
LPS12
97
ADSC
84
320
B
exosomes
CD63
CD9
75 kDa
25 kDa
25 kDa
ADSC LP6 LPS12
1 µg of exosomes / lane
C
miR-1246
miR-4532
miR-4454
miR-619-5p
miR-6126
Fold change
LP6
LPS12
ADSC

OS (n=22)		
Age (years)	Median (range)	14 (10-68)
Sex	Male	14
	Female	8
Tumor site	Trunk	2
	Extremity	20
Tumor state	Primary	21
	Recurrent	1
TNM	T1/T2/T3	4/15/3
	N0/N1	22/0
	M0/M1a/M1b	19/3/0
TNM stage	II A/IIB/ III/IVA	3/13/3/3

DDLPS (n=17)		
Age (years)	Median (range)	59 (38-77)
Sex	Male	13
	Female	4
Tumor site	Trunk	16
	Extremity	1
Tumor state	Primary	10
	Recurrent	7
TNM	T1/T2a/T2b	0/0/17
	N0/N1	17/0
	M0/M1	16/1
TNM stage	IB/ IIB/ III/IVA	1/3/12/1

EWS (n=3)			
No.	1	2	3
Age (years)	65	31	15
Sex	Female	Male	Male
Tumor site (Origin)	Trunk (Soft tissue)	Neck (Soft tissue)	Extremity (Bone)
Tumor state	Primary	Recurrent	Primary
TNM	T1bN0M0	T2bN0M0	T2N0M0
TNM stage	IIA	IIB	IIB