

Exosome-Derived miR-25-3p and miR-92a-3p Stimulate Liposarcoma Progression

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Abstract

Despite the development of combined modality treatments against liposarcoma in recent years, a significant proportion of patients respond only modestly to such approaches, possibly contributing to local or distant recurrence. Early detection of recurrent or metastatic disease could improve patient prognosis by triggering earlier clinical intervention. However, useful biomarkers for such purposes are lacking. Using both patient plasma samples and cell lines, we demonstrate here that miR-25-3p and miR-92a-3p are secreted by liposarcoma cells through extracellular vesicles and may be useful as potential biomarkers of disease. Both miR-25-3p and miR-92a-3p stimulated secretion of proinflammatory cytokine IL6 from tumor-

associated macrophages in a TLR7/8-dependent manner, which in turn promoted liposarcoma cell proliferation, invasion, and metastasis via this interaction with the surrounding microenvironment. Our findings provide novel and previously unreported insight into liposarcoma progression, identifying communication between liposarcoma cells and their microenvironment as a process critically involved in liposarcoma progression. This study establishes the possibility that the pattern of circulating miRNAs may identify recurrence prior to radiological detectability while providing insight into disease outcome and as a possible approach to monitor treatment efficacy. *Cancer Res*; 77(14); 3846–56. ©2017 AACR.

Introduction

Human liposarcoma is the most common soft-tissue sarcoma subtype, comprising 24% to 45% of all such forms of malignancy (1). Liposarcoma is classified into four subtypes: (i) well-differentiated LPS (WDLPS), (ii) de-differentiated LPS (DDLPS), (iii) myxoid/round cell LPS (MRC), and (iv) pleomorphic LPS (1).

WDLPS and DDLPS are characterized by chromosomal amplification at 12q13-q22, which contains the *MDM2* and *CDK4* genes (90% of the cases). In addition, genomic amplifications in 1p32, 1q21-24, and/or 6q23 and 13q-21-32 are frequently observed. WD/DDLPS is especially prevalent in the retroperitoneum (RLPS), where it has a devastating 10% 10-year overall survival rate. Surgical resection with negative margins remains the mainstay of definitive treatment for operable disease. However, high rates of local recurrence, coupled with acquisition of metastatic capacity in DDLPS (but not WDLPS), points to the need for even earlier therapeutic intervention than is currently possible due to limitations in detections of recurrence (1).

miRNAs are 19- to 24-nucleotide noncoding RNA molecules that regulate the expression of target mRNAs both at the transcriptional and translational level (2, 3). It has been observed that miRNA expression profiles may be predictively associated with different tumor types and stages in human cancers, potentially with even greater sensitivity than conventional methodologies (4, 5). miRNAs can be found in body fluids (i.e., blood serum or plasma) where they exist either cell-free, associated with proteins, or in blood-borne vesicles (6).

Circulating miRNAs are being investigated as potentially easily retrievable biomarkers for several diseases (7); more than 150 studies have assessed the potential use of serum or plasma miRNAs as biomarkers in different types of cancer (8). Furthermore, circulating miRNAs can convey epigenetic information, affecting gene expression in cells distant to the cellular miRNA source (9).

It has been demonstrated that secreted miRNAs can act in a paracrine manner in the surrounding microenvironment, promoting tumor development. For example, miR-21 and miR-29a are secreted by lung cancer cells and can bind human TLR8

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receptor (murine TLR7) in surrounding macrophages, triggering a NF- κ B-mediated prometastatic inflammatory response that could result in tumor growth and spread (10).

In this study we isolated extracellular vesicles (EV) from plasma patient samples and healthy controls. RNA samples were further profiled in order to both determine whether circulating miRNAs could serve as novel and specific potential biomarkers for liposarcoma and understand their functional significance in liposarcoma progression.

Materials and Methods

Patients and clinical samples

Blood samples of liposarcoma patients ($n = 24$) were collected from OSU James Cancer Medical Center (Columbus, OH), following the written informed consent in accordance with the Helsinki Declaration whose protocols have been approved by The Ohio State University Wexner Medical Center Institutional Review Board. Patient venous blood (12 mL) was collected in Vacutainer Plus whole blood tubes with K_2 EDTA (BD Biosciences). Blood plasma was retrieved from the whole blood samples via centrifugation at $1,900 \times g$ 10 minutes at 4°C , then aliquoted and stored at -80°C until analysis. Healthy donor blood used in the discovery and in the validation sets was purchased respectively from PrecisionMed and ZenBio. For plasma collection protocol, PrecisionMed centrifuged tubes at 2,440 rpm or $1,200 \times g$ for 10 minutes at room temperature, then plasma samples were stored at -80°C , whereas Zenbio spun blood samples at $1,300 \times g$ for 10 minutes at RT, then samples were stored at -20°C or colder temperature.

The detailed characteristics of patient and healthy control participants are summarized in Supplementary Table S1. Patient and control participants in the validation set are listed in Supplementary Table S2. Patient and control tissues analyzed are listed in Supplementary Table S3. Liposarcoma tumor tissues ($n = 24$: 9 matching blood samples and 15 new samples) and adjacent healthy tissues ($n = 6$) were also collected. Prior to any therapy, patient pathology was confirmed using surgically resected sarcomas, and graded as per standard Federation Nationale des Centres de Lutte contre le cancer (FNCLCC) criteria.

RNA isolation

Total RNA from plasma peripheral blood (PB) was isolated by using 400 μL of human plasma according to the manufacturer's protocol (miRCURY, Exiqon). RNA yield and purity was then determined using Nanodrop 1000 spectrophotometer (Thermo Scientific). Total RNA from tissue samples and from PBVs was isolated by using Norgen Kit and following the provided instructions (Norgen BioTek).

Peripheral blood vesicles were isolated from plasma by using ExoQuick (System Biosciences) and following manufacturer's protocol. The quality and size of particles was assessed through Nanosight (Supplementary Fig. S1). Vesicle RNA was then isolated by using Norgen Kit, as described above.

Quantitative real-time PCR

The expression level of an individual miRNA was determined using miRNA sequence specific probes (Thermo Fisher) as per quantitative real-time RT-PCR-based detection methodology. Total RNA was reverse transcribed by TaqMan (TaqMan Advanced miRNA cDNA Synthesis Kit; ThermoFisher) according to the

manufacturer's protocol. All samples were run in triplicate. RNU6 was used to normalize quantitative Real-Time PCR on RNAs extracted from cells. For vesicle-derived RNA normalization, ath-miR-159a, osa-miR-414, and cel-miR-248 synthetic oligos (Integrated DNA Technologies) were added to each sample. TaqMan Advance miRNA assays were used as follows: has-miR-199a-3p (PN002304), miR-25-3p (PN000403), miR-451a (PN001141), miR-92a-3p (PN000431; Thermo Fisher).

NanoString nCounter assay

The NanoString nCounter Human v3 miRNA Expression Assay has been used to perform the microRNA profiling analysis on RNA samples isolated from PB and PBVs of liposarcoma patients and healthy individual controls. The assay allows detecting and measuring the expression levels of up to 800 different microRNAs at the same time for each sample. Three microliters of RNA were annealed with multiplexed DNA tags (miR-tag) and bridges target specific. Mature miRNAs were bound to specific miR-tags using a ligase enzyme and all the tags in excess were then removed through the enzyme clean-up step. The tagged miRNA product was then diluted (ratio 1:5) and 5 μL were combined with 20 μL of reported probes in hybridization buffer and 5 μL of capture probes. The overnight hybridization (16–20 hours) at 65°C allowed to complex the probes sequence specific with targets. Probe excess was then removed using two-step magnetic beads based purification on an automated fluidic handling system (nCounter Prep Station) and target/probe complexes were immobilized on the cartridge for data collection. The nCounter Digital Analyzer collected the data by taking images of immobilized fluorescent reporters in the sample cartridge with a CCD camera through a microscope objective lens. For each cartridge, a high-density scan encompassing 600 fields of view was performed. Array values were scale normalized, using the sum of the control probe values to obtain a normalization factor for each profile.

Cell culture and transfection

Human liposarcoma cell lines Lipo224, Lipo246, and Lipo863 were established in our laboratory as previously reported (11). LPS141 was kindly provided by Dr. Jonathan Fletcher (Brigham and Women's Hospital). Liposarcoma cells were maintained using standard conditions and were grown in DMEM (Gibco), supplemented with 10% (v/v) FBS. U937 cells (ATCC) were cultured in RPMI (Gibco), supplemented with 10% (v/v) FBS. For differentiation, cells were incubated with 12-myristate 13-acetate (PMA) 100 ng/mL for 24 hours. Human HEK-Blue-293 (Invivogen) cells were cultured in DMEM supplemented with 10% (v/v) FBS, Normocin (50 $\mu\text{g}/\text{mL}$), Blasticidin (10 $\mu\text{g}/\text{mL}$), and Zeocin (100 $\mu\text{g}/\text{mL}$; Invivogen). Preadipocytes (XA15A1) were purchased from Lonza and maintained following the manufacturer's instructions. Murine Lewis lung carcinoma cells (LLC; ATCC) were cultured in RPMI1640, supplemented with 10% (v/v) FBS.

All the cell line used in this study were acquired within the past 5 years and authenticated by STR on February 9, 2017. Murine LLC cells were authenticated by morphology and biologic behavior. Preadipocytes and HEK-Blue-293 were bought within 6 months from this manuscript submission. Lonza tested the cells for differentiation and stained for adipocytes; HEK-Blue-293 were thoroughly tested and validated by InvivoGen. All cell lines were tested for mycoplasma.

EV isolation and treatments

EVs were isolated according to He and colleagues (12). The quality and the size of particles were assessed by Nanosight (Supplementary Fig. S2).

For experiments with synthetic miRNA oligos, cells were treated with HPLC-purified synthetic miRNAs (Integrated DNA Technologies) complexed with Dotap Liposomal Transfection Reagent (Roche), following the manufacturer's instructions. Briefly, synthetic miRNA oligos were complexed with Dotap reagent following the provided instructions, and incubated at RT for 15 minutes. The mixture was then added drop by drop to cells and the assay of interest was performed at the indicated time point.

For treatments with GW4869 (Sigma), Lipo246 cells were incubated with DMSO or GW4869 5 μ mol/L diluted in FBS-depleted medium for 48 hours; then EVs were isolated through ultracentrifugation.

Immunohistochemistry

Dissected tissues were fixed in 10% neutral-buffered formalin solution for 48 hours and transferred to 70% ethanol. Tissues were processed, embedded in paraffin, cut in 5- μ m sections on positively charged slides, deparaffinized, rehydrated, and stained with H&E. For IHC, all sections were stained using a Bond Rx autostainer (Leica). Briefly, slides were baked at 65°C for 15 minutes and automated software performed dewaxing, rehydration, antigen retrieval, primary antibody incubation, post primary antibody incubation, detection (DAB), and counterstaining using Bond reagents (Leica). Samples were then removed from the machine, dehydrated through ethanols and xylenes, mounted, and coverslipped. A RTU mouse antibody was used for CD68 (Leica).

LNA-anti-miRNA transfection

For transfection of liposarcoma cells with Exiqon, locked nucleic acid (LNA) negative control or miRCURY LNA inhibitor hsa-anti-miR-25-3p and hsa-anti-miR-92a-3p, Lipofectamine 2000 (Invitrogen) was used, following the manufacturer's instructions.

QUANTI-Blue assay

The QUANTI-Blue Assay (Invivogen) allows to verify whether a stimulus specific for human TLR8 receptor occurs through the activation of NF- κ B. For this purpose, HEK-Blue-TLR8 cells (Invivogen) were incubated with Dotap mixtures of synthetic miRNAs or with Lipo246-derived EVs for 24 hours, then QUANTI-Blue Assay was performed, according to the manufacturer's instructions.

Western blotting

For immunoblotting analysis cells were lysed with ice-cold NP-40 Cell Lysis Buffer (Invitrogen) plus protease inhibitors (Roche) for 30 minutes at 4°C. Equivalent amounts of protein were first mixed with sample buffer, then loaded on a Criterion Tris-HCl 4% to 20% pre-cast gel (Bio-Rad) and finally transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in Tris-buffered saline (pH 7.4) containing 0.05% Tween 20, then incubated with primary and secondary antibodies according to the manufacturer's instructions. Primary antibodies anti-phospho-p65 and anti-p65 (Cell Signaling Technology) were used, followed by isotype-matched, horseradish-peroxidase-con-

jugated secondary antibodies (GE Healthcare). Chemo-luminescence detection (Denville Scientific, Inc.) was finally performed.

Isolation of primary murine cells

Murine macrophages derived from the peritoneal cavity were isolated from age- and sex-matched WT C57/B6 mice and C57/B6 *TLR7*^{-/-} mice as previously described (10). Macrophages (300,000 cells) were stimulated with synthetic miRNAs or Lipo246-derived EVs for 48 hours, then conditioned media were collected and the ELISA assay for IL6 or TNF α were performed (Multi-Analyte ELISArray Kits, BD OptEIA), following the manufacturer's instructions.

MTS assay

Cell proliferation was assessed with 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTS)-Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega), following manufacturer's instructions. Metabolically active cells were detected by adding 20 μ L of MTS to each well. After 3 hours of incubation, the plate was analyzed in a Multilabel Counter (Bio-Rad Laboratories).

Migration

Cell migration was assessed by using transwell migration chamber (Corning). Briefly Lipo246 cells were seeded in the transwell upper chamber with macrophages supernatant with or without EVs. The lower chamber was filled with media supplemented with 10% FBS. After 24 hours, filters were washed, fixed, and stained with Coomassie Brilliant Blue (Sigma-Aldrich Corp.). Migrated cells in the lower surface of the filter were analyzed using Image J.

Invasion

Cell invasion through a 3D-extracellular matrix was assessed through BD Matrigel Invasion Chambers (BD Biocoat). Briefly, Lipo246 cells were seeded in the transwell upper chamber with macrophages supernatant with or without EVs. After 24 hours filters were washed, fixed, and stained with Coomassie Brilliant Blue (Sigma-Aldrich Corp.). Migrated cells to the lower filter were analyzed using Image J.

Statistical analysis

Differentially expressed miRNAs between comparison groups were determined by two-sided *t* tests and fold changes using log-transformed values. All ROC curves here were generated using ODS STATISTICAL GRAPHICS and LOGISTIC procedure statements in SAS 9.4. We also calculated the area under the ROC (AUC) of each ROC curve. AUC is the average sensitivity of the biomarker over the range of specificities that used as a summary statistic representing the overall performance of the biomarker. AUC of a biomarker with no predictive value would be 0.5, whereas a biomarker with an AUC of 1 would indicate perfect ability to predict disease.

Results

miR-25-3p and miR-92a-3p are secreted by liposarcoma cells through vesicles and may serve as potential biomarkers

To determine which miRNAs were secreted from liposarcoma cells and elucidate their possible role as prognostic "signatures," we performed a NanoString profiling on RNA samples isolated

from peripheral blood plasma vesicles (PBV) derived from 16 human liposarcoma patient samples and from eight healthy controls (Supplementary Table S1).

Figure 1A depicts a heatmap of the most differentially expressed PBVs miRNAs (102 miRNAs). However, when we profiled the total peripheral blood (PB), only 54 miRNAs were consistently deregulated (Fig. 1B). Moreover, the miRNAs consistently deregulated in PB (fold change > 3) were also observed as miRNAs significantly deregulated in the PBVs (Fig. 1C), meaning that most of the miRNAs deregulated in the PB belong to the PBV compartment. For this reason, we decided to concentrate our study on the PBV miRNA panel. We next performed a quantitative RT-PCR to validate our results in a new cohort of liposarcoma samples (Supplementary Table S2). This analysis confirmed a differential expression for four miRNAs; three were found to be upregulated (miR-25-3p, miR-451a, miR-92a-3p) whereas one was downregulated (miR-199a-3p; Fig. 1D).

Many members of the miR-17-92 cluster, a well-known oncogenic cluster (13), were found deregulated in the array. This cluster is in a region often amplified in liposarcoma (chr13q31.3) and correlates with a poor prognosis (14).

To determine a possible tumor cell origin, we then evaluated whether the miR-17-92 cluster members and miR-25-3p were secreted into the culture media through EVs by different liposarcoma cell lines. Interestingly, only miR-25-3p and miR-92a-3p were upregulated in the EVs derived from different liposarcoma cell lines compared to preadipocytes (Fig. 2A–G), indicating that they may have been of tumor origin. To confirm that miR-25-3p and miR-92a-3p were secreted by tumor cells through vesicles, we isolated EVs from plasma patient samples and healthy controls ($n = 3$) using a different protocol (ultracentrifugation), confirming the results obtained by using cell lines, as both microRNAs were differentially expressed (Supplementary Fig. S3A). Moreover, to further demonstrate that miR-25-3p and miR-92a-3p were EV-associated, we incubated Lipo246 cells for 48 hours with GW4869 5 $\mu\text{mol/L}$, an inhibitor of small vesicle secretion that also impairs the content of vesicle-secreted miRNAs (15). We noticed that the expression levels of both miRNAs were strongly impaired when Lipo246 cells were incubated with GW4869 with respect to the control, supporting our previous findings (Supplementary Fig. S3B).

Interestingly, when we assessed the expression levels of miR-25-3p and miR-92a-3p in the liposarcoma tumor tissues, we found that they were downregulated (Supplementary Fig. S3C). This inverse relationship has been found in various cancers (16); based on our results, we propose that the presence of miRNAs in the circulation may reflect the miRNA composition of the tumor microenvironment.

ROC curve analyses were conducted in the discovery sets to estimate the sensitivity and specificity for circulating miR-25-3p and miR-92a-3p in discriminate liposarcoma patients from controls. The AUC for miR-25-3p was 0.86 and for miR-92a-3p was 0.82, indicating a separation between the liposarcoma cancer group and the healthy control group in our discovery cohort (Fig. 2H and I), and thereby supporting their correlation with tumor diagnosis.

EV-secreted miR-25-3p and miR-92a-3p are involved in the communication between tumor cells and the surrounding microenvironment

We next asked whether secreted miR-25a-3p and miR-92a-3p had a role at the microenvironment level by possibly impacting

on liposarcoma growth. miRNAs secreted through EVs have been shown to be involved in the communication between tumor and the surrounding microenvironment in some systems and may have a key role in the tumor growth by acting as hormones in a paracrine manner (10).

IHC performed on liposarcoma patient tissue samples revealed the presence of infiltrating macrophages (Fig. 3 and Table 1). We therefore stimulated peritoneal macrophages derived from wild-type (WT) C57/B6 mice using either Dotap (Roche) mixtures of miR-25-3p and miR-92a-3p or Lipo246-derived EVs. After 48 hours, an ELISA assay showed that synthetic miRNAs specifically promoted the secretion of IL6 from macrophages, whereas Dotap itself or Dotap formulation of miR-16 had no effect on cytokine secretion. Similar to LLC-derived EVs, Lipo246-secreted EVs also induced IL6 release from macrophages (Fig. 4A).

To verify whether IL6 secretion induced by Lipo246-derived EVs occurred in a TLR7/8-dependent manner, we isolated peritoneal macrophages from *TLR7*^{-/-} mice and we treated cells either with Dotap mixture of synthetic miR-25-3p or miR-92a-3p or with Lipo246-secreted EVs, as previously described. Interestingly, IL6 secretion from *TLR7*^{-/-} macrophages was strongly impaired, both when cells were incubated with Dotap formulations of synthetic miR-25-3p and miR-92a-3p or when cells were incubated with Lipo246-secreted EVs, suggesting that murine TLR7 receptor is required for this process (Fig. 4B). We also incubated WT C57/B6-derived peritoneal macrophages with EVs derived from the plasma of healthy donors (C1, C3, C7) and liposarcoma patients (10, 11, 12, 15, 19, 20, 21, 39; detailed characteristics on patients and healthy control participants are summarized in Supplementary Tables S1, S2, and S3). As expected, liposarcoma-derived EVs promoted the secretion of IL6 from macrophages, whereas this process was strongly impaired in the healthy donor counterpart, therefore corroborating our previous finding (Fig. 4C), $P < 0.03$.

Finally, we treated WT C57/B6-derived peritoneal macrophages for 48 hours with EVs isolated from Lipo246 cells previously transfected with locked nucleic acid (Exiqon): LNA-anti-scrambled oligomer, LNA-anti-miR-25-3p or LNA-anti-miR-92a-3p. The ELISA results strongly supported our other data in that IL6 secretion were impaired when miRNAs were silenced with respect to control (Fig. 4D).

In light of these findings, we asked if murine TLR7 receptor-mediated secretion of IL6 from macrophages occurred through the NF- κ B pathway. Genetically modified HEK-293 cells (HEK-Blue-TLR8 cells; Invivogen) overexpressing human TLR8 receptor were treated with Dotap mixtures of miR-25-3p and miR-92a-3p or with Lipo246-derived EVs. Similar to Dotap formulation of miR-21, which was used as a positive control (10), both miR-25-3p and miR-92a-3p induced the activation of the NF- κ B pathway mediated by human TLR8 (Fig. 4E and Supplementary Fig. S4). In contrast, Dotap alone or Dotap mixture of miR-16 had a strongly reduced effect on NF- κ B activation (Fig. 4E). These data were supported by the treatments of genetically modified HEK-293 with Lipo246-isolated EVs. EVs derived from cells that had been previously transfected with LNA-anti-scrambled oligomer induced the activation of the NF- κ B pathway. On the contrary, when miR-25-3p and miR-92a-3p were silenced in Lipo246 cells, the incubation of genetically modified HEK-293 cells with liposarcoma-derived EVs strongly impaired the NF- κ B activation (Fig. 4F).

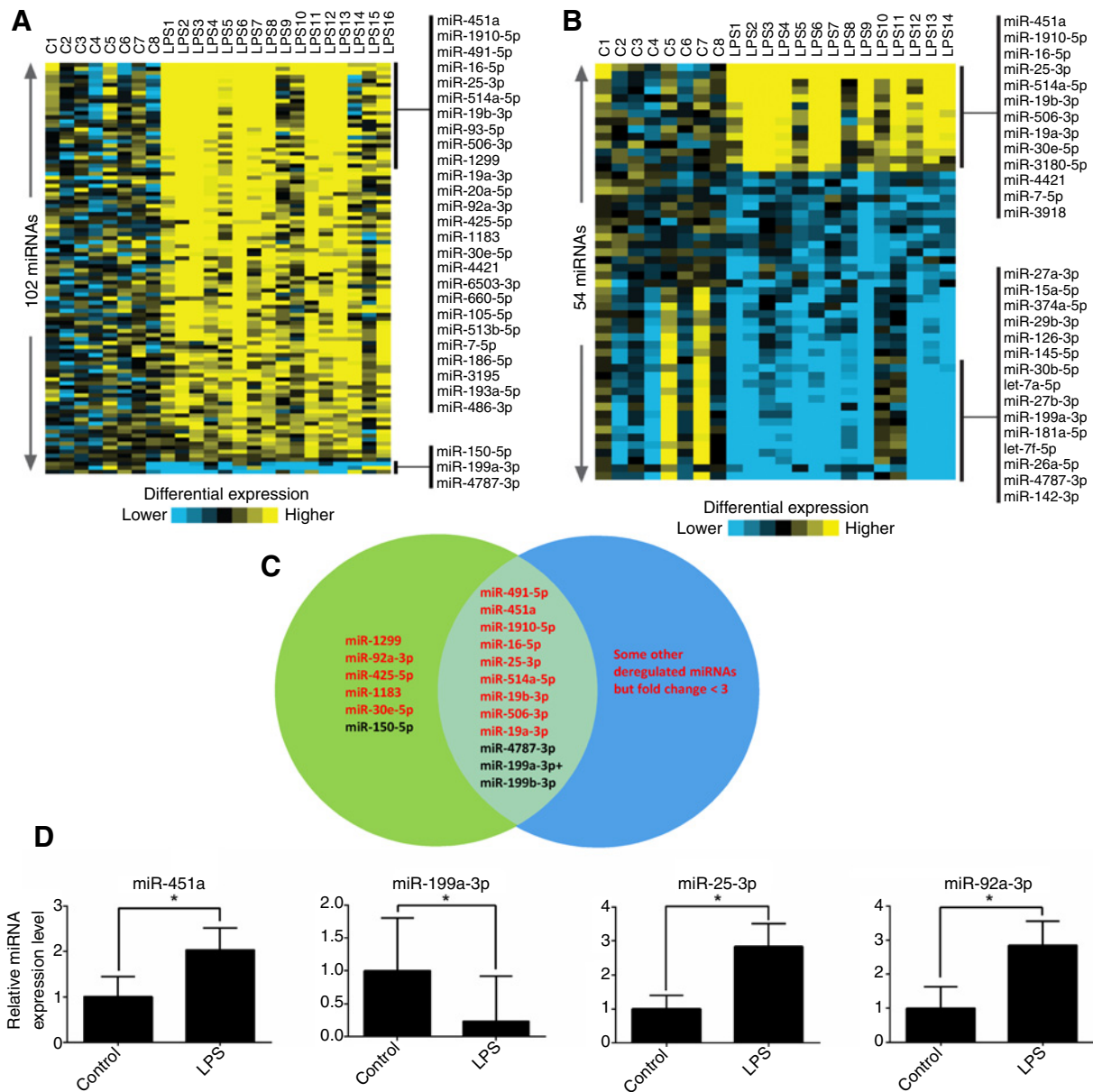


Figure 1. miRNA expression signature of PBVs and PB in liposarcoma (LPS) patient samples. **A** and **B**, Heatmap representation of the top deregulated miRNAs in LPS-PBVs (**A**) and LPS-PB (**B**) compared with healthy donor. Each row represents the relative levels of expression for a single miRNA and each column shows the expression levels for a single sample. The yellow or blue color indicates relatively high or low expression, respectively (differences significant with $P < 0.01$). **C**, Venn diagram reveals the overlap of deregulated miRNAs in PBVs (green) compared with PB (blue). Red, upregulated miRNAs in liposarcoma; black, downregulated miRNAs in liposarcoma. **D**, Validation of selected miRNAs performed through qRT-PCR showing significant differential expression levels ($P < 0.05$) in an independent cohort of liposarcoma samples (Supplementary Table S2; $N = 3$).

EV-stimulated secretion of IL6 promotes in turn liposarcoma proliferation, migration, and invasion

To determine the effect of macrophage-secreted IL6 on the tumor itself, we considered whether this cytokine could affect tumor growth. Human macrophages were incubated for 48 hours with Lipo246-derived EVs or Dotap mixtures of miR-25-3p or miR-92a-3p. We then incubated Lipo246 cells with macrophage-conditioned medium for 72 hours and assessed cell proliferation, migration, and invasion. Incuba-

tion with the supernatant of macrophages previously incubated with EVs resulted in increased Lipo246 proliferation with respect to control (Fig. 5A). The same results were achieved when the supernatants of macrophages previously incubated with Dotap formulation of synthetic miR-25-3p and miR-92a-3p were used (Fig. 5B). When the migration and invasion assays were performed on Lipo246 cells incubated with the same conditions as described above, it was possible to show that these processes were promoted by the supernatant of

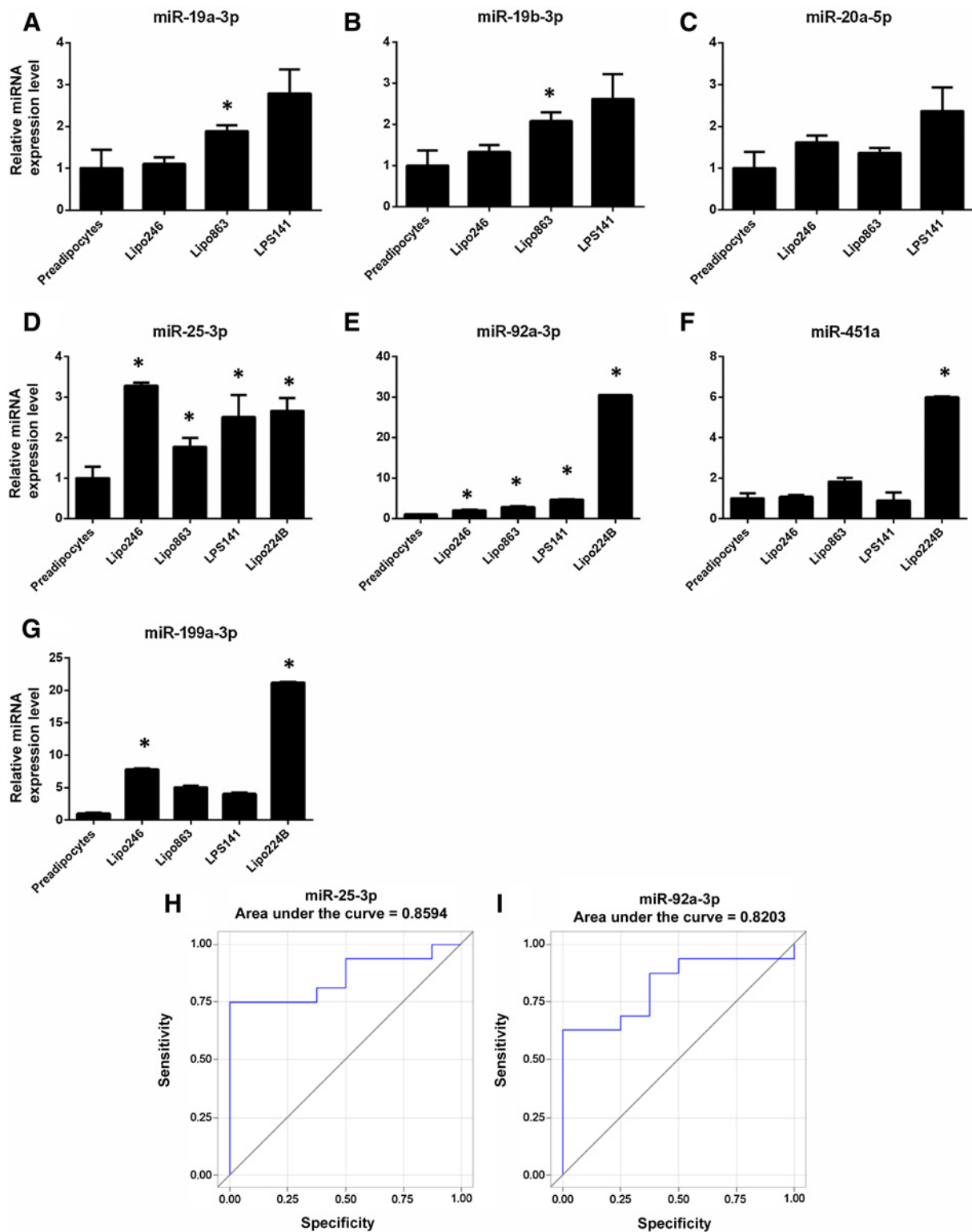


Figure 2. miR-25-3p and miR-92a-3p are secreted from liposarcoma (LPS) cell lines through EVs. EVs were secreted from different liposarcoma cell lines (Lipo246, Lipo863, LPS141, Lipo224B) or normal preadipocytes, then different miRNA expression levels were assessed and compared within isolated fractions. The expression levels of miR-25-3p and miR-92a-3p were significantly higher in EVs released from liposarcoma cell lines with respect to EVs derived from normal preadipocytes (D and E; $P < 0.05$ for miR-25-3p and $P < 0.003$ for miR-92a-3p). On the contrary, miR-451, miR-199a-3p, miR-19a-3p, and miR-19b-3p, miR-20a-5p expression levels were not significantly different for all cell-derived EVs (A, B, C, F, and G). H and I, ROC curve analysis using PBVs miR-25-3p (H) and miR-92a-3p (I; $N = 3$).

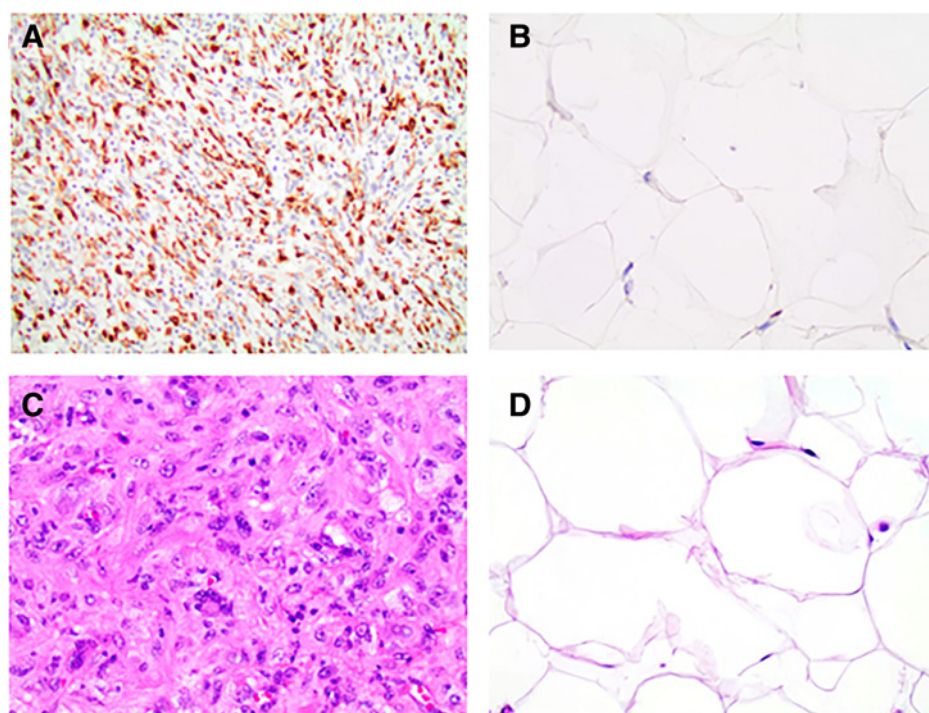


Figure 3. Liposarcoma induces high level of macrophages infiltration. **A–D**, Representative images of immunohistochemical staining human liposarcoma ($\times 100$; **A**) and normal fat ($\times 200$; **B**) using anti-CD68 antibody. H&E for liposarcoma (**C**) and normal fat (**D**) are shown ($\times 100$).

macrophages previously incubated with EVs (Fig. 5C and D). These findings suggest that secreted miR-25-3p and miR-92a-3p promote IL6 secretion from surrounding macrophages, leading to liposarcoma growth in a paracrine manner.

Discussion

In this study we have demonstrated that circulating miRNA-25-3p and miRNA-92a-3p can serve as novel and specific potential biomarkers for liposarcoma. Moreover, we showed that miR-25-3p and miR-92a-3p impact the surrounding microenvironment in that liposarcoma-derived EVs stimulate the secretion of IL6 from macrophages in a TLR7/8-dependent fashion.

We also showed that IL6 secretion occurs through the NF- κ B pathway and determined that macrophage-secreted IL6 promotes liposarcoma growth in a feedback loop. Figure 6 depicts a mechanism summarizing these findings incorporating possible cross-talk between liposarcoma and surrounding immune cells. Our study provides previously unreported insights into

liposarcoma progression, identifying the importance of communication between liposarcoma cells and their microenvironment in liposarcoma progression.

Several previous studies have shown that circulating miRNAs can serve as biomarkers for diagnosis of various cancers (16, 17). In sarcoma, miRNA profiling in tumor tissue may be useful for tumor characterization, in particular miR-155, miR-21, miR-26a-2, miR-13, miR-145, miR-144/451 in liposarcoma (18–23). However, to the best of our knowledge, a study reporting an array analysis characterizing circulating miRNAs in liposarcoma has not yet been described. Previously, Fricke and colleagues identified a blood-borne miRNAs signature in synovial sarcoma (24). Recently Boro and colleagues suggested that miR-155 might be a diagnostic marker for DDLPS, but in his work only five DDLPS patients were analyzed (25). Moreover, his analysis was only restricted to a list of miRNAs found to be deregulated in the DDLPS tissues, whereas an analysis of the entire blood miRNA panel was lacking. Some miRNAs deregulated in our array were also found in the literature to be deregulated in DDLPS tissues (18, 26). miR-155 was actually not found to be significantly deregulated in our samples, even though it was previously shown to be upregulated in DDLPS tissues (22, 25). The discrepancy existing between this and other studies may be explained by interpatient variability that may be relevant when low sample numbers are analyzed.

EVs have recently become the focus of intensive scientific research as novel mediators of intercellular communication. Several studies have recently established that miRNAs secreted by cancer cells through EVs are involved in the communication between the tumor and the surrounding microenvironment. To the best of our knowledge, no liposarcoma cell lines have been shown to release EVs, and the potential for dialogue between EVs and liposarcoma cells has not been evaluated and no role for miRNAs in the liposarcoma microenvironment have been heretofore considered. Here

Table 1. CD-68 immunopositivity was scored semiquantitatively for the percent of tumor cells staining

ID	Histology	Percent of tumor cell stained	Intensity
LPS-1	DDLPS	60%	3
LPS-2	DDLPS	90%	3
LPS-10	DDLPS	80%	3
LPS-13	DDLPS	80%	3
LPS-32	DDLPS	70%	2–3+
LPS-19	WDLPS	25%	2–3
LPS-20	WDLPS	30%	3
LPS-41	WDLPS	20%	2–3
LPS-42	WDLPS	40%	3

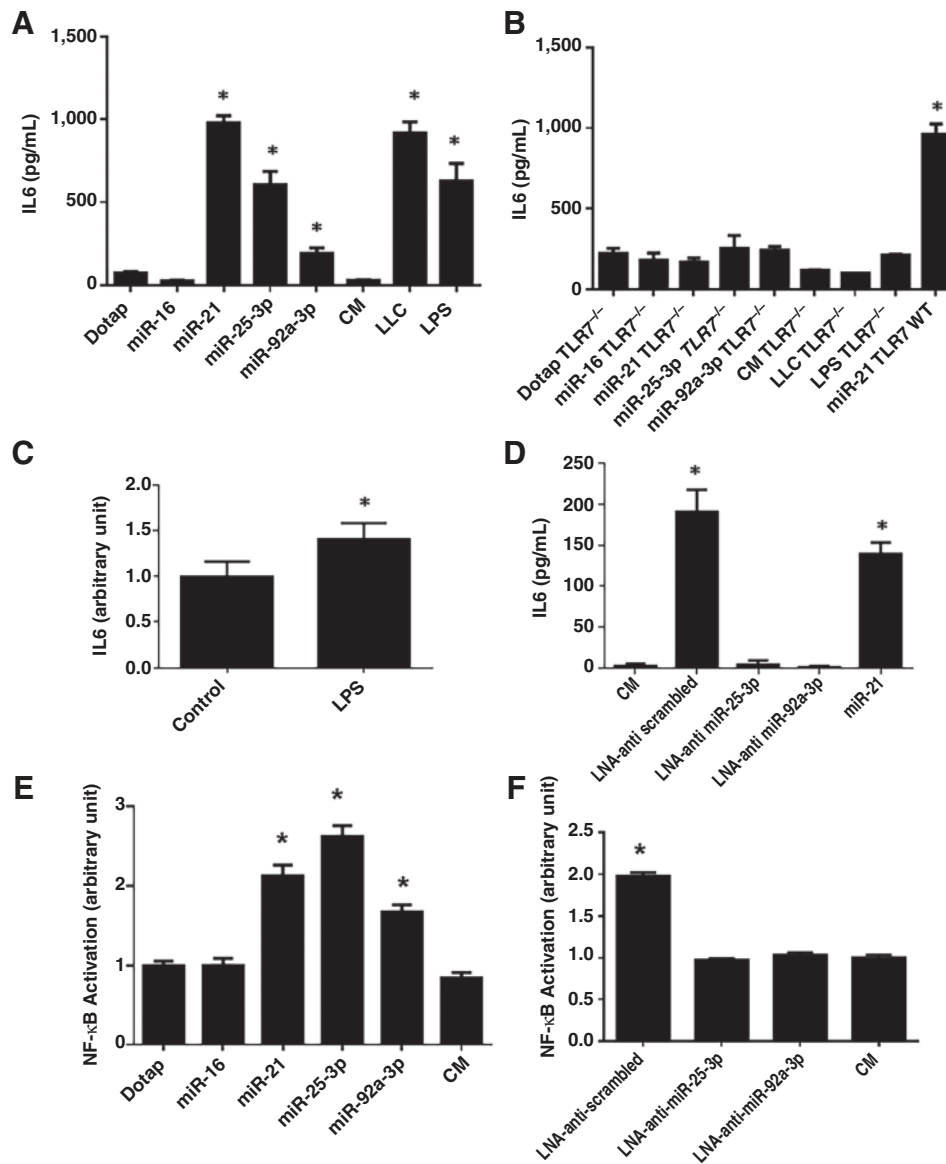


Figure 4.

miR-25-3p and miR-92a-3p stimulate IL6 secretion from macrophages via NF-κB in a TLR7/8-dependent manner. **A**, ELISA assay performed on peritoneal macrophages isolated from WT C57/B6 mice ($n = 4$) and treated with Dotap mixture of the indicated miRNAs and with Lipo246-secreted EVs (LPS). As positive controls, the complex Dotap-miR-21 and LLC-derived EVs were used. As negative control, the complex Dotap-miR-16 and Lipo246 conditioned medium (CM) were used. *t* test: for EVs, all conditions vs. conditioned medium ($P < 0.0005$); for synthetic oligos, all conditions vs. miR-16 ($P < 0.0008$). **B**, Secretion of IL6 from macrophages isolated from *TLR7*^{-/-} mice was impaired either when cells were treated with Lipo246-derived EVs or either when they were incubated with Dotap formulations of miR-25-3p and miR-92a-3p. As a positive control for the assay, the supernatants derived from macrophages isolated from WT mice or stimulated with the complex Dotap-miR-21 were used ($P < 0.05$). **C**, ELISA assay for IL6 performed on human macrophages incubated for 48 hours with EVs isolated from the plasma of liposarcoma (LPS) patients and healthy controls. Error bar, SE; $P < 0.03$. **D**, ELISA assay for IL6 showing the specific stimulation of murine macrophages by miR-25-3p and miR-92a-3p. When peritoneal macrophages were incubated with Lipo246-derived EVs, previously transfected with LNA-anti-miR-25-3p and LNA-anti-miR-92a-3p, the secretion of IL6 was strongly impaired ($P < 0.0003$) with respect to control (EVs derived from Lipo246 transfected with LNA-antisense scrambled). **E**, TLR8-HEK-Blue-293 cells were incubated with Dotap mixtures of miR-25-3p and miR-92a-3p for 24 hours. Dotap formulation of miR-21 was used as a positive control, whereas Dotap itself, Dotap formulation of miR-16, or cells incubated with Lipo246-CM was used as a negative control. Finally, QUANTI-Blue assay was performed. For miR-21, miR-25-3p and miR-92a-3p Dotap treatments vs. miR-16 Dotap treatment. $P < 0.001$. **F**, TLR8-HEK-Blue-293 cells were also incubated for 24 hours with EVs derived from Lipo246 cells previously transfected with LNA-antisense scrambled, LNA-anti-miR-25-3p, or LNA-anti-miR-92a-3p. Incubation with CM was used as a negative control. *t* test was performed for all conditions vs. incubation with CM ($P < 0.001$).

we have shown that liposarcoma cells release EVs, and their content has functional significance, contributing to liposarcoma cell proliferation through NF-κB pathway.

The involvement of NF-κB in liposarcoma has been demonstrated only in the myxoid liposarcoma subtype (27). Activation of the NF-κB pathway has been proposed to explain

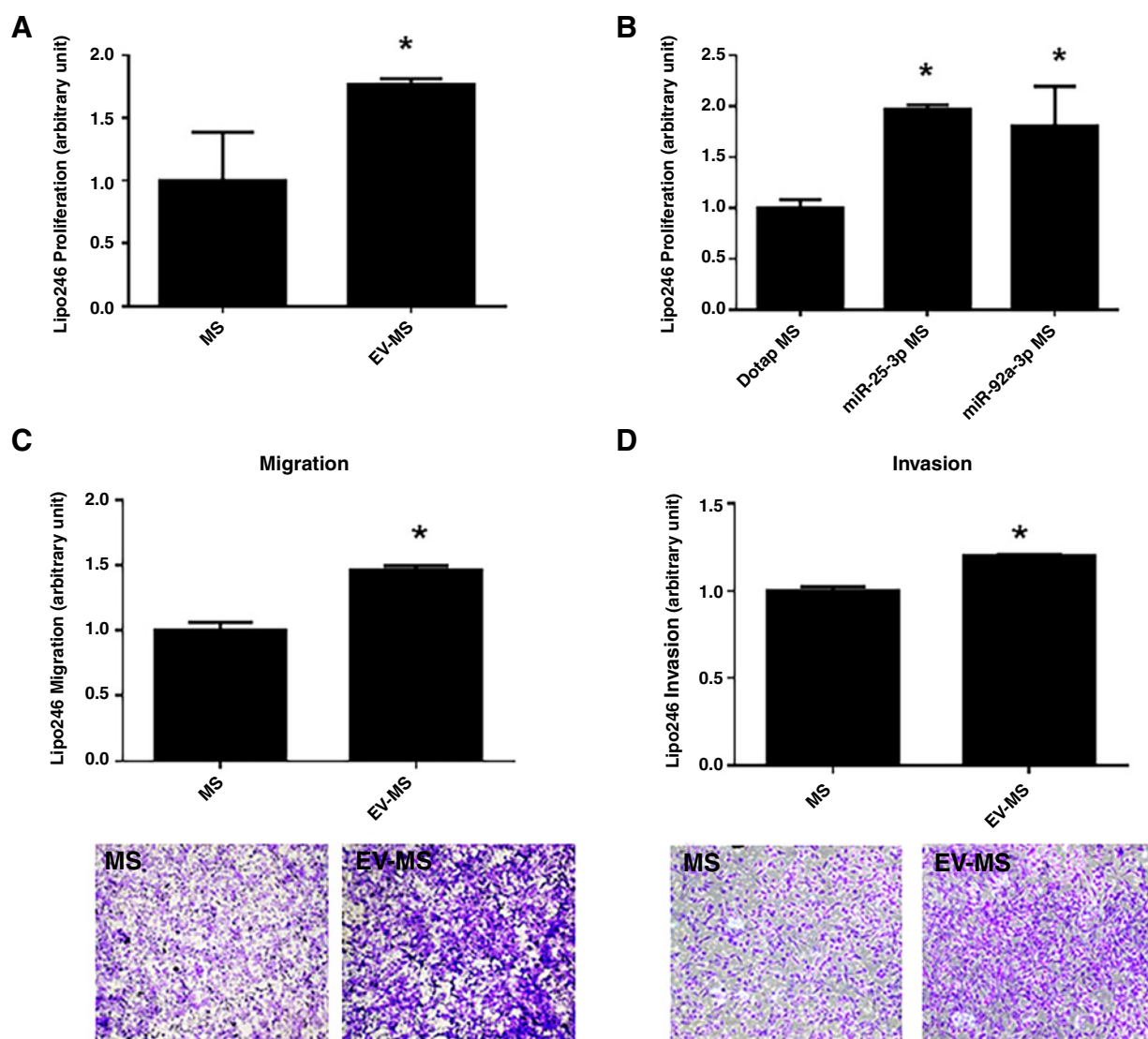


Figure 5.

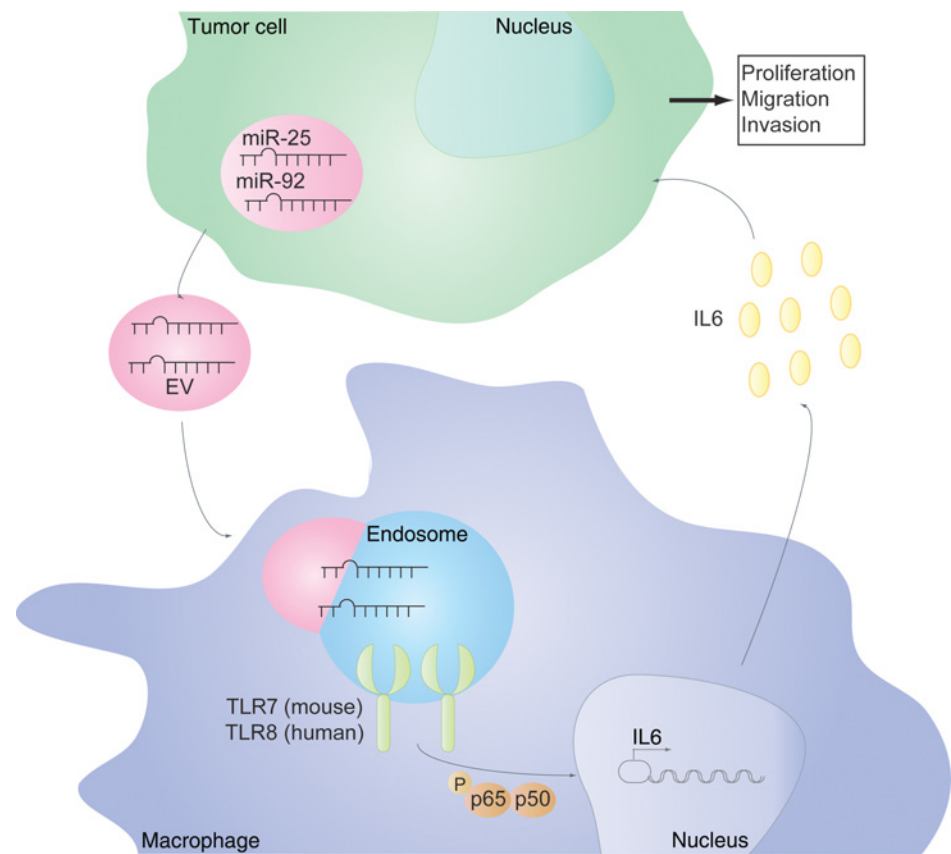
Macrophage-secreted IL6 promotes liposarcoma cell proliferation, migration, and invasion. Lipo246 cells were treated with conditioned medium derived from differentiated U937 macrophages previously incubated for 48 hours with Lipo246-derived EVs. Tumor cell proliferation, migration, and invasion were then assessed: **A**, MTS assay showing Lipo246 proliferation after 72 hours of incubation with macrophagic supernatant derived from treatment with Lipo246-secreted EVs. **B**, the same assay was performed by using macrophagic supernatant derived from treatments with the indicated synthetic oligos (*, $P < 0.05$). **C**, Cell migration assay performed on Lipo246 incubated with macrophagic supernatant derived from conditioned medium or treatment with Lipo246-EVs. Top panel shows quantification ($N = 3$), whereas bottom panel shows representative images. *, $P < 0.0005$. **D**, Invasion assay performed on Lipo246 cells treated as described in **C**. Top panel shows quantification ($N = 3$), whereas bottom panel shows representative images. *, $P < 0.0002$. MS, macrophagic supernatant derived from treatment with conditioned medium; EV-MS, supernatant derived from macrophages previously incubated with Lipo246-derived EVs; Dotap-MS, supernatant derived from macrophages previously incubated with Dotap; miR-25-3p-MS/ miR-92a-3p-MS, supernatant derived from macrophages previously incubated with Dotap mixture of miR-25-3p/ miR-92a-3p.

HOXA5-induced apoptosis in DDLPS (28). Here we found that NF- κ B is involved in liposarcoma tumor development in that either miR-25-3p or miR-92a-3p stimulates the immune cell secretion of the proinflammatory cytokine IL6 in a TLR7/8-dependent manner via the NF- κ B pathway.

Cancer-related inflammation is now recognized as a tumor hallmark (29–31). Chronic inflammation is implicated in nearly all stages of tumorigenesis and, in particular, IL6 is involved in cancer development (32, 33). In sarcoma, it has

been shown that IL6 plays a pivotal role in proliferation and/or invasion in myxoid liposarcoma and osteosarcoma (34–36). IL6 has been found to be involved with drug resistance in osteosarcoma (37). Such data suggest the importance of IL6 in the liposarcoma tumor microenvironment and are consistent with our findings. Our data also show that liposarcoma-derived EVs stimulate also the secretion of TNF α from macrophages in a TLR7/8-dependent manner (Supplementary Fig. S5). Comprising another proinflammatory cytokine, we can

Figure 6. Model of the mechanism proposed in this study for miR-25-3p and miR-92a-3p in the tumor microenvironment. miR-25-3p and miR-92a-3p stimulate macrophages to release IL6. In a feedback loop, IL6 stimulates the tumor growth, promoting cell proliferation, invasion, and migration.



speculate that $\text{TNF}\alpha$ may also contribute to liposarcoma growth and spread; further investigations are ongoing.

This study was able to address a gap in knowledge about crosstalk between liposarcoma and the microenvironment; we propose a not previously reported role for $\text{NF-}\kappa\text{B}$ and IL6 in liposarcoma growth involving miR-25-3p and miR-92a-3p. Moreover, we have begun to address the urgent need for biomarkers in this burdensome disease. A limitation of our study is the low number of patients examined. However, liposarcoma is a rare malignancy that is further subdivided into at least four different subtypes, thus making the assembly of a large number of patients within one institutional study cohort difficult. To address this issue, a large validation study involving multiple institutions is already being undertaken by our group. This initial study may therefore be useful as preliminary to subsequent research efforts that will consider the pattern of circulating miRNAs before and after surgery. These future efforts may help to establish whether these miRNAs can be useful to identify recurrence prior to radiological detectability, predict disease outcome, or possibly monitor efficacy of treatment. Based on these studies, further exploration of miRNAs as therapeutic targets for liposarcoma appears warranted as a potential new treatment for patients burdened by this understudied yet devastating form of malignancy.

In conclusion, in this study we have established that circulating vesicular miR-25-3p and miR-92a-3p can reliably distinguish between liposarcoma patients and healthy controls. These findings suggest the possibility that circulating vesicular miRNAs could serve as novel, noninvasive biomarkers whose

signatures may prove invaluable in the early diagnosis of liposarcoma, while offering more accurate prognosis and even prediction of recurrence. As functional candidates, we propose that miR-25-3p and miR-92a-3p participate in the protumoral inflammatory process by activating the TLRs response of immune cells, leading to liposarcoma growth in a paracrine manner.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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