



MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer

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Abstract

Objectives. Most ovarian cancer patients are diagnosed at an advanced stage (67%) and prospects for significant improvement in survival reside in early diagnosis. While expression patterns of a recently identified biomarker family, microRNA, appear to be characteristic of tumor type and developmental origin, microRNA profiling has been limited to tissue specimens. Tumors actively release exosomes into the peripheral circulation and we now demonstrate the association of microRNAs with circulating tumor-derived exosomes.

Methods. Circulating tumor exosomes were isolated using a modified MACS procedure with anti-EpCAM. Initially, microRNA profiles of ovarian tumors were compared to those of tumor exosomes isolated from the same patients. Levels of 8 microRNAs (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214) previously demonstrated as diagnostic, were compared in exosomes isolated from sera specimens of women with benign disease and various stages of ovarian cancer.

Results. MicroRNA from ovarian tumor cells and exosomes from the same patients were positive for 218 of 467 mature microRNAs analyzed. The levels of the 8 specific microRNAs were similar between cellular and exosomal microRNAs (exhibiting correlations from 0.71 to 0.90). While EpCAM-positive exosomes were detectable in both patients with benign ovarian disease and ovarian cancer, exosomal microRNA from ovarian cancer patients exhibited similar profiles, which were significantly distinct from profiles observed in benign disease. Exosomal microRNA could not be detected in normal controls.

Conclusions. These results suggest that microRNA profiling of circulating tumor exosomes could potentially be used as surrogate diagnostic markers for biopsy profiling, extending its utility to screening asymptomatic populations.

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Keywords: MicroRNA; Ovarian cancer; Diagnosis; Screening; Exosomes

Introduction

Despite progress made in the understanding and treatment of ovarian cancer, it remains the sixth most common cancer in women worldwide, causing approximately 125,000 deaths annually [1]. Most women with ovarian cancer are diagnosed at an advanced stage, with 75% diagnosed with extra-ovarian disease [2]. In comparison with other cancers associated with women, 73% of endometrial cancers, 55% of breast cancers and 50% of cervical cancers are diagnosed with Stage I disease [3].

While the 5-year survival of patients with Stage I ovarian cancer exceeds 90%, only 21% of advanced-stage ovarian cancer patients survive 5 years after initial diagnosis [2]. Since long-term survival has not changed significantly in the last two decades, the best prospects for further improvement in ovarian cancer survival reside in early diagnosis [3].

Over the last 5 years, expression profiling technologies have identified new biomarkers with diagnostic applications. One such biomarker group is a class of small noncoding RNAs, termed microRNAs [4–6]. MicroRNAs, small (22–25 nucleotides in length) noncoding RNAs, suppress the translation of target mRNAs by binding to their 3' untranslated region [7,8]. Post-transcriptional silencing of target genes by microRNA can occur either by cleavage of homologous mRNA or by specific inhibition of protein synthesis. MicroRNAs are critical regulators of cellular

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processes, such as proliferation, differentiation, development, and cell death [9]. All tumors analyzed by microRNA profiling have exhibited significantly distinct microRNA signatures, compared with normal cells from the same tissue [4,6,10]. Lu et al. [11] performed an analysis of leukemias and solid cancers and determined that microRNA-expression profiles could classify human cancers by developmental lineage and differentiation state. The expressions of individual microRNAs and specific microRNA signatures have now been linked to the diagnosis and prognosis of many human cancers.

Using tissue specimens, Iorio et al. [4] demonstrated that, in comparison to normal ovary, specific microRNAs were aberrantly expressed in ovarian cancer, with *miR-141*, *miR-200a*, *miR-200b*, and *miR-200c* being the most significantly overexpressed. They further demonstrated the hypomethylation in ovarian tumors resulted in the up-modulation of *miR-21*, *miR-203*, and *miR-205*, compared with normal ovary. Two of these up-modulated microRNAs, *miR-200a* and *miR-200c*, were enhanced in all the three histologic types examined (serous, endometrioid, and clear cell), whereas *miR-200b* and *miR-141* up-modulation was shared by endometrioid and serous histologic types. In general, the microRNA signatures obtained comparing different histologic types of ovarian cancers (serous, endometrioid, clear cell, and mixed) with the normal tissue were overlapping in most cases. Their analysis of ovarian tumors also demonstrated the absence of differentially expressed microRNAs in relation to tumor stage or grade, which could have resulted from their set of samples being primarily derived from advanced-stage tumors. However, they also postulated that microRNAs might be critical for the development of ovarian cancer, but not for its progression [4].

Among the microRNAs most significantly up-modulated, *miR-200a* and *miR-141* belong to the same family, *miR-200b* is localized on chromosome 1p36.33 in the same region as *miR-200a* and *miR-200c* is localized on chromosome 12p13.31 in the same region of *miR-141* [4]. This association would agree with the findings of Zhang et al. [12] that proposed that the up-modulation of specific microRNAs could be the amplification of the microRNA genes. Using high-resolution array-based comparative genomic hybridization, an aberrantly high proportion of loci containing microRNA genes exhibited DNA copy number alterations. In ovarian cancer, 37.1% of the genomic loci containing microRNA genes were associated with DNA copy number alterations [12]. In breast cancer and melanoma, an even greater proportion of these loci exhibit altered DNA copy numbers (72.8% and 85.9%, respectively) [12]. As a result, microRNA-expression patterns, or signatures, appear to be more characteristic of the developmental origins of tumors than mRNA expression patterns and may be associated with diagnosis, staging, progression, prognosis, and response to treatment. However, as cancer diagnostic tools, the analyses of microRNA signatures are limited to tissue biopsies.

In 1979, we initially demonstrated the presence of tumor-derived exosomes, small (50–100 nm) membrane vesicles of endocytic origin, in the peripheral circulation of women with ovarian cancer [13,14]. Since our original report, other cells have been demonstrated to be capable of releasing exosomes, including reticulocytes, dendritic cells, B cells, T cells, mast cells,

epithelial cells, and embryonic cells [15,16]; however, their accumulation in the peripheral circulation appears to be unique to cancer and pregnancy [17,18]. While the primary source of circulating exosomes in cancer patients is the tumor, other normal cells within the peripheral circulation can contribute to the level exosome population. Our recent work has focused on the separation of tumor-derived exosomes from those derived from normal lymphoid cells [19,20]. Utilizing adherence to specific magnetic beads, exosomes of tumor origin can be isolated.

Since exosome functionality appears to be determined by its specific protein content, proteomic analysis has been performed on *in vivo* and *in vitro* derived exosomes. Analyses of exosomes have demonstrated that all exosomes share certain common characteristics, including structure (lipid bilayer), size, density and general protein composition. Some proteins are commonly associated with all exosomes, including cytoplasmic proteins (such as tubulin, actin, actin-binding proteins, annexins and Rab proteins), signal transduction proteins, (protein kinases, heterotrimeric G-proteins), MHC class I molecules, and heat-shock proteins (such as Hsp70 and Hsp90) [21–24]. Tetraspanins, including CD9, CD63, CD81 and CD82, are the protein family most commonly associated with exosomes and are generally used as exosome markers [23,25]. While tumor-derived exosomes exhibit some common, shared proteins, they also express an array of tumor antigens that reflect the originating tumor cells. Although exosome release can be demonstrated in many proliferating cell types, their release is exacerbated in tumor cells, as evidenced by their elevated presence in plasma, ascites and pleural effusions of cancer patients [26,27]. This elevated presence in serum and ascites fluids of cancer patients and the overexpression of certain biomarkers has lead investigators to propose a role for exosomes in diagnosis and biomarker analysis [28].

Exosomes have been postulated to play an important role in cell–cell communication and appear to affect target cells either by stimulating them directly by surface expressed ligands or by transferring molecules between cells. Ratajczak et al. [29] demonstrated the presence of exosomal RNA and provided evidence for the horizontal transfer of genetic information between cells. The biological effects of these exosomes were inhibited after pretreatment with RNase, indicating the involvement of RNA components. RNA molecules, following translocation from the nucleus to the cytoplasm, can bind to and be transported by membranous organelles or vesicles to specific intracellular sites, which may provide an explanation for the association of RNA populations with exosomes. Valadi et al. [30] demonstrated that released exosomes contain a subset of both cellular mRNA and microRNA, which could be transferred to target cells. Our preliminary results suggest that microRNA contained in tumor exosomes is functional and can suppress the mRNA for signal transduction components within T cells. Since released exosomes contain RNA populations, including microRNA, it is possible that this exosomal microRNA reflects the microRNA signature of the parental tumor. The objectives of this study were to determine whether the microRNAs contained within ovarian cancer-derived exosomes mirrored that of the tumor and thus could be used diagnostically.

Materials and methods

Patient samples and cell lines

This study utilized sera derived from women diagnosed with serous papillary adenocarcinoma of the ovary ($n=50$; $n=10$ for Stage I, $n=10$ for Stage II, $n=20$ for Stage III and $n=10$ for Stage IV), age-matched women with benign ovarian adenoma ($n=10$), and age-matched women with no evidence of ovarian disease ($n=10$). Controls, patients with benign ovarian disease and Stages III and IV ovarian cancer were selected based on age-matching to patients with early stage ovarian cancer. This study also investigated primary tumor cell cultures, established from 6 women with Stage IIIc cyst adenocarcinoma of the ovary, and their corresponding pre-surgery sera samples. All of these materials were obtained under an informed consent approved by the University Human Studies Committee of the University of Louisville. The primary ovarian tumor cell cultures were established in our laboratory and designated UL-1, UL-2, UL-3, UL-6, UL-B, and UL-O. UL-2 and UL-3 were derived from hereditary ovarian cancers, while UL-1, UL-6, UL-B, and UL-O were derived from spontaneous cancers. These ovarian tumor cells were grown in RPMI 1640 medium supplemented with 10% exosome-free (by ultrafiltration) fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 200 mM L-glutamine, 100 mg/ml streptomycin and 100 IU/ml penicillin in a humidified 5% CO₂ atmosphere. Cell viability was evaluated by trypan blue exclusion and all cultures utilized were >95% viable.

Isolation of circulating exosomes

Tumor-derived exosomes were specifically isolated by a modified magnetic activated cell sorting (MACS) procedure, using anti-epithelial cell adhesion molecule (EpCAM). Our previous studies have demonstrated that exosomes from epithelial tumors express EpCAM on their surface and can be used for their selective isolation. Serum samples (2.5 ml) from normal controls, patients with benign disease, and patients with early stage ovarian cancer were incubated with anti-EpCAM coupled to magnetic microbeads (50 μ l). These were mixed and incubated for 2 h at 4 °C. A LD microcolumn was placed in the magnetic field of a MACS Separator and the column was rinsed with 500 μ l Tris-buffered saline (TBS). The magnetic immune complexes were applied onto the column and unbound (unlabeled) material passed through and was discarded. The column was washed four times with 500 μ l of TBS. The specifically selected exosomes were recovered by removing the column from the separator and placing it on a collection tube. TBS (1 ml) was added to the column and the magnetically labeled exosomes were obtained by applying the plunger supplied with the column. The isolated exosomes/microbeads were diluted in IgG elution buffer (Pierce Chemical Co, Rockford, IL) and the complex was centrifuged at 10,000 rpm to separate the microbeads from the exosomes (supernatant). The supernatant was then centrifuged at 100,000 g for 1 h at 4 °C. The pelleted exosomes were resuspended in 250 μ l phosphate-buffered saline (PBS) and these tumor-derived exosomes were assayed for total protein. The quantity of protein was determined by the Bradford microassay method (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin (BSA) as a standard.

Transmission electron microscopy

For transmission electron microscopy, the pelleted exosomes were fixed in 2.5% (w/v) glutaraldehyde in PBS, dehydrated and embedded in Epon. Ultrathin sections (65 nm) were cut and stained with uranyl acetate and Reynold's lead citrate. The sections were examined in a Jeol 1210 transmission electron microscope.

Isolation and profiling of microRNA

Total RNA was isolated from the tumor cells and exosomes using the mirVana microRNA isolation kit according to manufacturer's instructions (Ambion, Austin, TX). The RNA quality, yield, and size of microRNA fractions were analyzed using Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA). The isolated microRNAs were 3'-end labeled with Cy3 using the mirVana microRNA Array Labeling Kit (Ambion) and the Post Labeling Reactive Dye kit

(Amersham Bioscience, Pittsburgh, PA). MicroRNA profiling was performed in duplicate by Ocean Ridge Biosciences (Jupiter, FL) using microarrays containing probes for 467 human mature microRNAs. This analysis used custom-developed microRNA arrays covering the 467 microRNAs present in the Sanger Institute mirBASE v9.0, consisting of 35–44-mer oligonucleotides, manufactured by Invitrogen and spotted in duplicate. After hybridization, the microRNA arrays were scanned using a GenePix 4000A array scanner (Axon Instruments, Union City, CA) and the raw data normalized and analyzed using GeneSpring 7.0 Software (Silicon Genetics, Redwood City, CA). Normalization was performed by expressing each microRNA replicate relative to control microRNA (Ambion) added to each sample, allowing comparisons between arrays. Threshold and 95th percentile of negative controls (TPT95) were calculated based on hybridization signal from negative control probes including: 38 mismatch and shuffled control probes and 87 non-conserved *Caenorhabditis elegans* probes. To define sensitivity, NCode synthetic microRNA was spiked at 1/500,000 mass ratio into labeling reactions and the signal intensity was detected. For specificity, perfect match probes for miR-93, miR-27a, and miR-152 and 2 mismatches for each were used. The 2 base pair mismatch probes demonstrated a signal below or at TPT95 on all arrays.

To assess the stability of the exosomal profiling with storage and manipulations, sera from patients with ovarian cancer patients were obtained and aliquoted into four 4 ml samples. Tumor exosomes were isolated from the first aliquot by the MACS procedure immediately and total RNA was isolated and stored at –70 °C until isolation of all samples. The remaining sera samples were stored at 4 °C for subsequent exosome isolation. Tumor exosomes were isolated from the second aliquot after 24 h, from the third aliquot after 48 h and from the fourth sample after 96 h at 4 °C. RNA was isolated from each exosome preparation and stored. In a similar study, 3 additional serum aliquots were stored at –70 °C for 7 to 28 days, prior to exosome and RNA isolations to mimic the use of banked specimens.

General statistical considerations

Data were analyzed using the statistical software package, SAS9.1 (SAS Institute, Cary, NC). The levels of circulating exosomes for each group of patients were defined as means \pm standard deviations from at least two separate experiments performed in triplicate. Comparisons between these groups were performed by one-way ANOVA, followed by the Tukey's multiple comparisons post-test comparing each population. Relative quantification of microRNA expression was calculated with the 2– $\Delta\Delta$ Ct method (Applied Biosystems User Bulletin No. 2) and data were analyzed as log₁₀ of relative quantity (RQ) of the target microRNA, normalized with respect to control microRNA added to each sample, allowing comparisons between arrays. The microRNA distributions and correlations along with confidence intervals were calculated for each subset. Statistical significance was set as $p \leq 0.05$.

Results

Presence of circulating EpCAM-positive exosomes in women with benign and malignant ovarian disease

EpCAM-positive exosomes were specifically isolated using anti-EpCAM magnetic beads and these circulating exosomes were assayed for total protein and plotted versus stage of disease (Fig. 1A). The levels of EpCAM-positive exosomes in age-matched normal volunteers (control) were 0.039 ± 0.030 mg/ml of exosomal protein, which represented the background of the assay. Patients diagnosed with benign ovarian disease possessed 0.149 ± 0.065 mg/ml of exosomal protein, which was significantly elevated over controls. Patients diagnosed with ovarian cancer all exhibited significantly elevated levels of EpCAM-positive exosomes (compared to benign disease or controls). Women with Stage I ovarian cancer exhibited 0.320 ± 0.056 mg/ml of circulating exosomal protein, which was significantly greater than

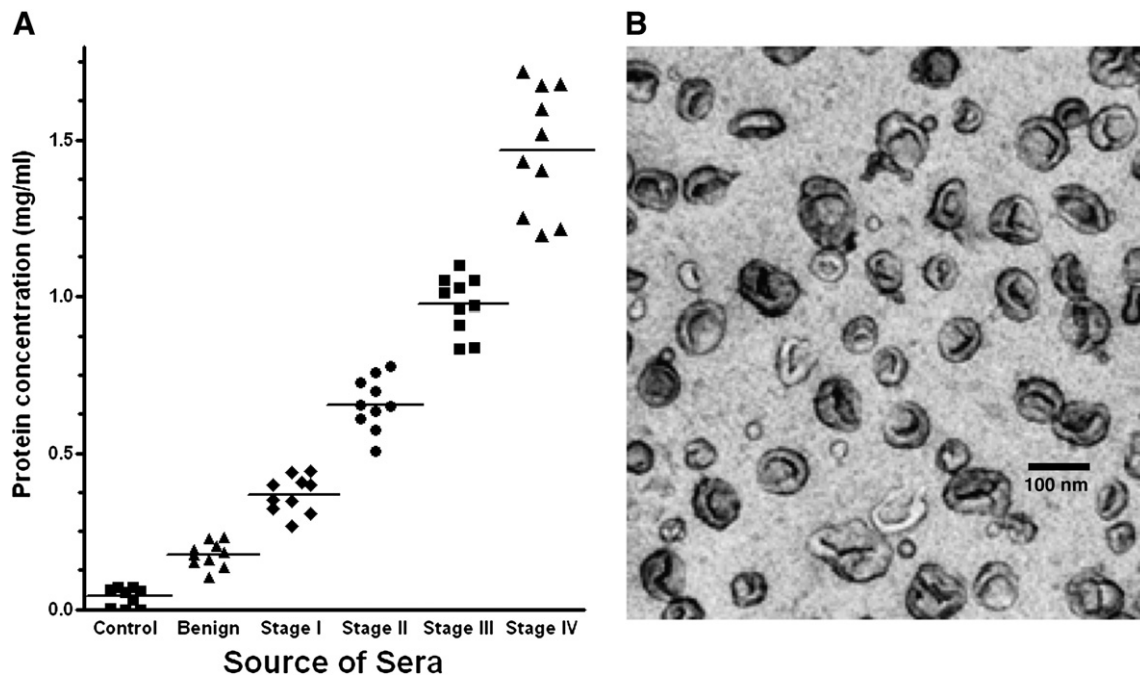


Fig. 1. Panel A: The levels of circulating tumor-derived exosomes compared to stage of ovarian cancer. Exosomes were isolated from sera obtained from age-matched female controls ($n=10$), age-matched women with benign ovarian disease ($n=10$), and women diagnosed with ovarian cancer ($n=10$ for each stage). Levels of exosomes are presented as protein concentrations. Panel B: Electron micrograph of circulating exosomes isolated by magnetic beads. Ultrathin sections (65 nm) were cut and stained with uranyl acetate and Reynold's lead citrate. The sections were examined in a Jeol 1210 transmission electron microscope.

both controls and benign disease ($p<0.01$). The levels of circulating exosomes increased as the stage progressed, with Stage II cancer having 0.640 ± 0.053 mg/ml, Stage III possessing 0.995 ± 0.084 mg/ml and Stage IV presenting with 1.42 ± 0.228 mg/ml. Levels of exosomes associated with these three stages were significantly greater than women with benign disease or controls

($p<0.001$). The resulting fractions were further analyzed by electron microscopy, which demonstrated vesicular structures characteristic of exosomes (Fig. 1B). The exosomal nature of this material was further confirmed by the presence of tetraspanins, class I antigens, and placental-type alkaline phosphatase by Western immunoblotting (data not shown).

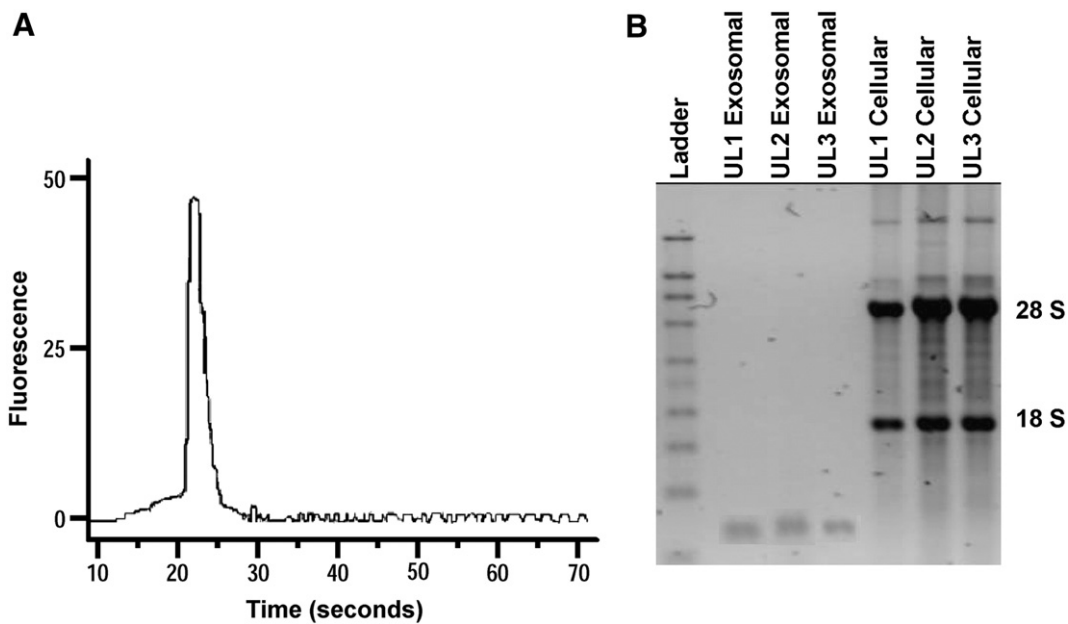


Fig. 2. Presence of small RNA associated with circulating EpCAM-positive exosomes from ovarian cancer patients. Panel A: Representative analysis of the RNA isolated from tumor exosomes using Agilent 2100 Bioanalyzer. Panel B: Agarose gel (1%) separation of total RNA from circulating exosomes and corresponding tumors. This total RNA was used as the starting material for microRNA profiling.

Association of small RNA with tumor-derived exosomes

To identify whether these isolated exosomes contained small RNAs, they were examined using a Bio-Analyzer 2100 (Fig. 2). This analysis identified the presence of a significant population of small RNA in the absence of 18S and 28S RNA, generally observed with cell-derived RNA. This material was subsequently used for microRNA profiling.

Profiling of exosome-derived versus cell-derived microRNA

The presence and levels of specific microRNAs from both cell-derived and exosome-derived microRNA were determined using microarray analysis probing for 467 microRNAs. The microRNA profiles of our ovarian tumors confirmed the alterations, previously reported [4]. Further, we demonstrated that of the 467 microRNAs, 218 were above the normalized threshold, calculated based on the 95th percentile of the negative control probe signal in both the cells and exosomes (Table 1). Of the 218 positive microRNAs, the levels of 175 were not significantly different between the ovarian tumor cells and their corresponding exosomes. By comparison, 12 were present at a higher proportion in the cells, while 31 were present at elevated levels within exosomes.

Previously, specific microRNAs were demonstrated to be overexpressed in human ovarian cancer (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, and miR-214). To correlate these findings with exosomal-derived material, RNA fractions were isolated from the original tumor cells and circulating tumour exosomes of the same patients (Fig. 3). Using microarray analysis, comparisons between tumor-derived microRNA profiles and peripheral blood-derived exosomal microRNAs indicated that they were not significantly different.

Further, the levels of tumor-derived microRNA profiles exhibited a strong correlation with the levels of peripheral blood-derived exosomal microRNAs (for miR-21, $r=0.77$; miR-141, $r=0.88$; miR-200a, $r=0.76$; miR-200b, $r=0.85$; miR-200c, $r=0.83$; miR-203, $r=0.85$; miR-205, $r=0.91$; and miR-214, $r=0.71$).

Exosomal microRNA correlation with presence and stage of disease

Our previous comparisons between tumor and circulating exosomes were performed with advanced-stage patients. To compare the associations of specific microRNAs with the presence of disease across various stages, the mean intensities of exosomal microRNAs were determined. The presence of the 8 diagnostic microRNAs among patients with Stages I, II and III were not significantly different for most of these miRNAs (Fig. 4). miR-200c and miR-214 were lower in patients with Stage I, compared to Stages II and III. However, in all cases, these miRNAs were significantly elevated over the levels detected in exosomes derived from benign disease. The small RNA fraction could not be demonstrated in normal controls and attempts to assess the presence of miRNAs were negative.

Stability of exosomal microRNA profiles

Since the measurement of circulating exosomal microRNA appears to be diagnostic, the technical question of its stability was raised. When the microRNA profiles were performed on serum samples stored over short time periods at 4 °C (up to 96 h) and the intensities compared (Fig. 5A), no significant differences were observed in the 3 diagnostic microRNAs analyzed. When the serum samples were stored at – 70 °C for longer time intervals, the

Table 1
Association of microRNA with peripheral blood-derived tumor exosomes compared with microRNA isolated from their corresponding tumors

Elevated in cells	Equal between cells and exosomes	Elevated in exosomes
miR-218, miR-196a, miR-195, miR-15a, miR-519d, miR-382, miR-503, miR-34b, miR-520d, miR-29c, miR-135a, miR-155	miR-296, miR-20a, miR-28, miR-302a, miR-99a, miR-99b, miR-10a, let-7a, let-7b, let-7c, let-7d, let-7f, let-7g, let-7i, miR-138, miR-23a, miR-183, miR-25, miR-107, miR-181a, miR-125a, miR-222, miR-198, miR-16, miR-200a, miR-18a, miR-101, miR-136, miR-31, miR-106b, miR-92, miR-342, miR-128a, miR-182, miR-663, miR-502, miR-500, miR-652, miR-424, miR-130a, miR-429, miR-365, miR-29a, miR-550, miR-422a, miR-585, miR-92b, miR-629, miR-671, miR-210, miR-26a, miR-454-5p, miR-769-3p, miR-765, miR-301, miR-191, miR-93, miR-200b, miR-100, miR-324-5p, miR-220, miR-151, miR-186, miR-128b, miR-130b, miR-125b, miR-122a, miR-30d, miR-203, miR-15b, miR-192, miR-133a, miR-126, miR-98, miR-190, miR-137, miR-105, miR-96, miR-95, miR-519b, miR-29b, miR-453, miR-23b, miR-517c, miR-625, miR-200c, miR-193a, miR-22, miR-224, miR-369-3p, miR-106a, miR-181c, miR-17-5p, miR-19b, miR-24, miR-17-3p, miR-221, miR-335, miR-126, miR-181a, miR-331, miR-188, miR-9, miR-34a, miR-30c, miR-19a, miR-371, miR-10b, miR-21, miR-148a, miR-339, miR-187, miR-346, miR-146a, miR-185, miR-328, miR-196b, miR-129, miR-522, miR-30a-5p, miR-27a, miR-30a-3p, miR-494, miR-20b, miR-521, miR-181b, miR-423, miR-487b, miR-425-3p, miR-594, miR-532, miR-512-3p, miR-526a, miR-578, miR-638, miR-422b, miR-484, miR-486, miR-645, miR-146b, miR-571, miR-647, miR-637, miR-30b, miR-452, miR-361, miR-432, miR-375, miR-766, miR-768-3p, miR-769-5p, miR-513, miR-362, miR-565, miR-30e-3p, miR-320, miR-590, miR-152, miR-181d, miR-660, miR-584, miR-141, miR-18b, miR-582, miR-505, miR-628, miR-425-5p, miR-421, miR-27b, miR-768-5p, miR-454-3p, miR-148b, miR-194, miR-345, miR-26b	miR-214, miR-140, miR-147, miR-135b, miR-205, miR-150, miR-149, miR-370, miR-206, miR-197, miR-634, miR-485-5p, miR-612, miR-608, miR-202, miR-373, miR-324-3p, miR-103, miR-593, miR-574, miR-483, miR-527, miR-603, miR-649, miR-18a, miR-595, miR-193b, miR-642, miR-557, miR-801, let-7e

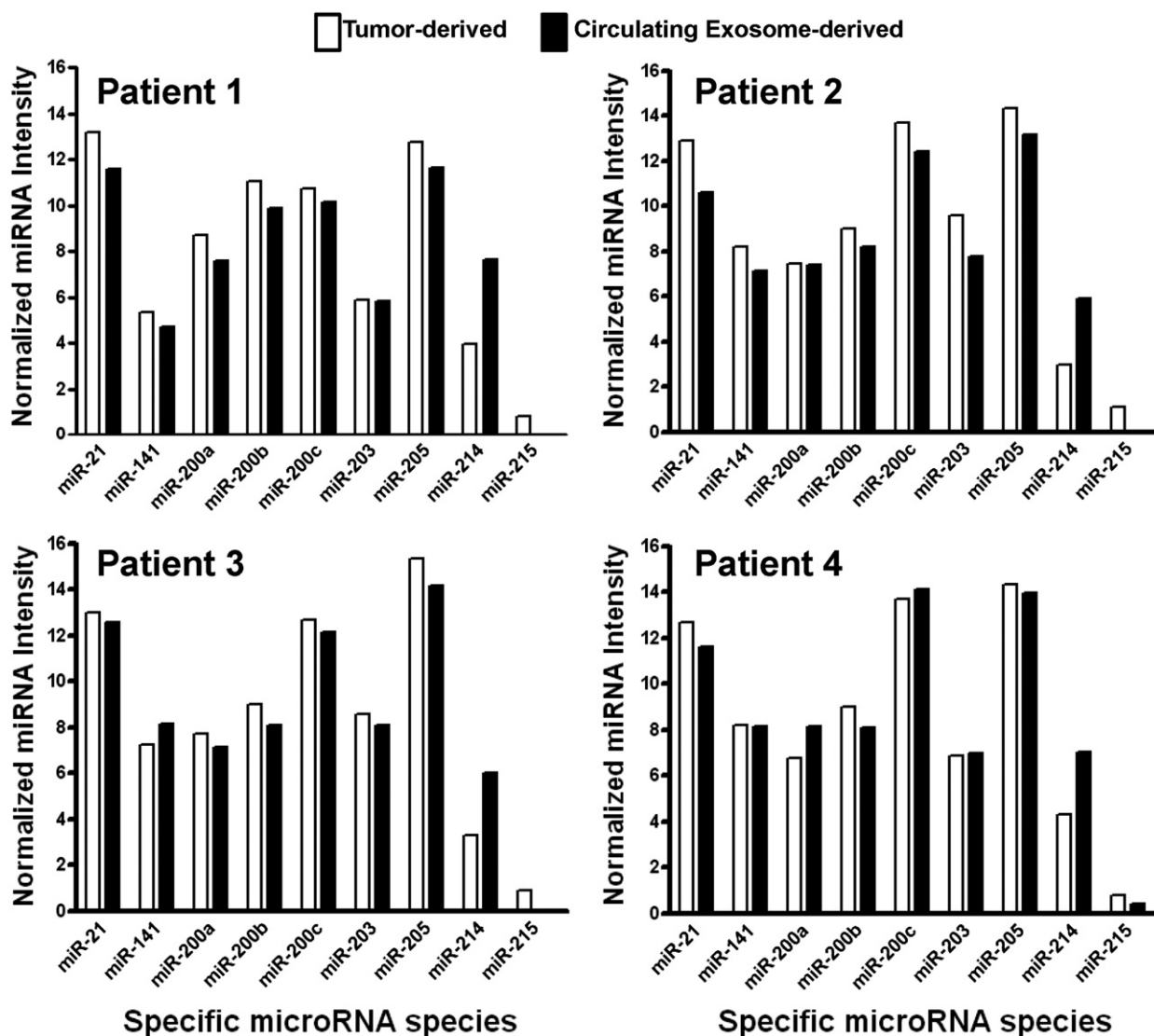


Fig. 3. Intensities for specific microRNAs derived from the advanced-staged ovarian tumors (□) and from EpCAM-positive exosomes (■) isolated from the sera of these patients. miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, and miR-214 have previously been demonstrated to be upregulated markers for ovarian cancer. Each bar presents the average intensities of duplicate samples with the results of four representative patients presented.

intensities of these microRNAs on the microarrays were not significantly different (Fig. 5B). These results indicate that the levels of these exosomal microRNAs were stable and do not significantly change with storage.

Discussion

MicroRNA-expression profiling can be used as diagnostic tools for cancers that currently lack reliable molecular markers, such as ovarian cancer. While previous studies have indicated that microRNA signatures could serve as diagnostic and prognostic markers for ovarian cancer, these data were based on their expression in tissue specimens. Here, we report the association of microRNA with circulating tumor-derived exosomes. In previous studies, microRNAs have been demonstrated to be aberrantly expressed in human ovarian cancers and the overall microRNA expression could differentiate normal versus cancer

tissues [4]. The study of Lu et al. [11] demonstrated the use of microRNA signatures as an important advance in cancer diagnosis. Their work indicated that microRNA-based identification of cancers was superior in correctly diagnosing cancer of unknown primaries than mRNA classification. However, it is currently not possible to use microRNA profiling in the absence of a mass to be biopsied.

Our original electron microscopic characterization of exosomes indicated that they were hollow (i.e. absence of viral-like structures) [17]. As a result, our group, together with others, focused on external protein components of exosomes and the biologic consequences of exosome exposure. In the present study, we demonstrate for the first time the presence of small RNA species associated with circulating tumor exosomes (Fig. 2). This small RNA lacks the 18S and 28S associated with RNA from cells. Further, microarray analysis demonstrated that at least part of the small RNA identified is microRNA.

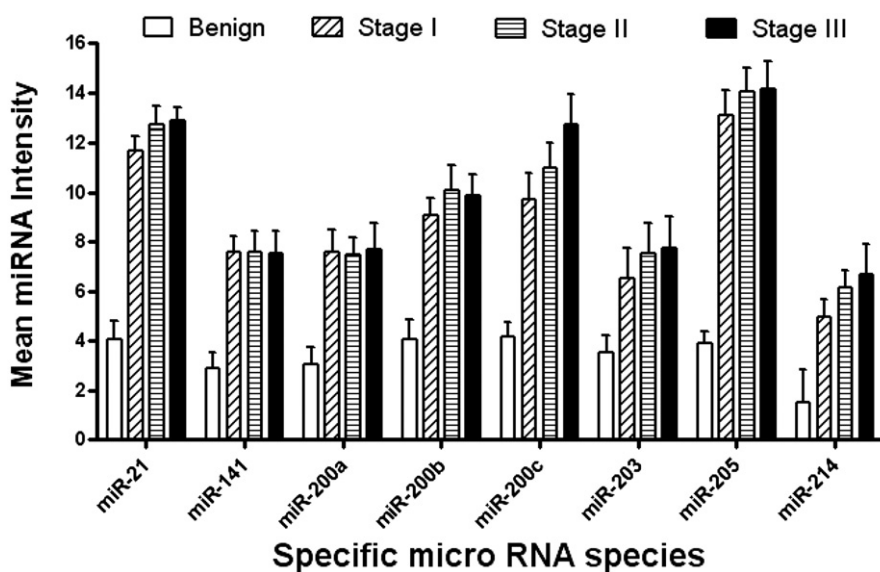


Fig. 4. Intensities for specific microRNAs derived from EpCAM-positive exosomes isolated from the peripheral blood (2.5 ml) of the patients with benign ovarian disease and patients with ovarian cancer. Patients with ovarian cancer were separated between Stages I, II, and III. The bars represent the mean \pm standard deviation of the normalized intensities of each group of patients ($n=10$ for each group).

The microRNA-expression profiles of our ovarian tumor cells confirmed the microRNA aberrations reported in previous studies. Analyses of both circulating tumor exosomes and the tumor cells from the same patients demonstrated that both were positive for 46% of the tested microRNAs (218/467). When the intensities of the microRNA were normalized, most of these microRNAs were expressed at similar levels between the cells and exosomes or were elevated within the exosomes (175 were not significantly different and 31 were elevated within exosomes). Thus, the aberrantly expressed microRNAs, used to establish cancer-specific signatures, appear in both cellular and exosomal compartments of ovarian cancer patients.

Our comparison of specific microRNAs, previously demonstrated to be diagnostic, indicated a high degree of correlation between the microRNA from the tumor and its corresponding exosomes (ranging from 0.71 to 0.90). This high correlation even holds for microRNAs that appeared to be present at higher proportions in exosomes, such as for miR-214. The uniform elevation of specific microRNAs in exosomes has led to the suggestion that compartmentalization of microRNAs into to exosomes, for at least some microRNAs, is an active (selective) process. Such a process could be mediated by components, such as nucleolin or nucleophosmin, which are aberrantly expressed on tumor exosomes.

Since these results suggest that exosomal microRNA profiling could be used as a surrogate for tissue microRNA and the goal in screening would be the identification of early stage disease, the ability to detect circulating exosomal microRNAs in early stage disease was examined. The exosomal microRNA expressions of the diagnostic microRNAs between patients with early versus late stage ovarian cancers were not significantly different for most of these microRNAs (Fig. 4). miR-200c and miR-214 were lower in patients with Stage I, compared to Stages II and III; however, in all cases, these microRNAs were significantly elevated over the levels detected in exosomes derived from benign disease. The small RNA fraction could not be demonstrated in normal controls

and attempts to assess the presence of microRNAs were negative. Thus, the absence of exosomes and/or exosomal small RNA is associated with normal, non-cancer-bearing individuals and exosomal microRNA mirroring normal tissue microRNA profiles appear to be associated with benign disease. The similarity across the stages of ovarian cancer is likely the result of standardization of starting exosomal small RNA quantities and the normalization of the resulting array data. Despite this standardization and normalization, the profiles obtained with exosomal microRNA from patients with benign disease remained distinct. There are several issues that are not analyzed in this pilot study. Since previous studies examining the microRNA signatures obtained comparing different histologic types of ovarian cancers (serous, endometrioid, clear cell, and mixed) failed to demonstrate differentially expressed microRNAs in terms of tumor stage or grade, our proof of concept study focused on only serous papillary adenocarcinomas and did not address differences in grade. Further, beyond the 8 microRNAs identified by Iorio et al. [4], some microRNAs appear to be differentially expressed between early stage ovarian cancer and late stage disease; however, a larger scale study including additional confounding factors will need to be performed to define their significance. Patients with benign ovarian disease were symptomatic and referred to the division for resection of the ovarian mass. Thus, it is unclear what the profile of women with asymptomatic masses would be. The women constituting the control group, in addition to not having cancer, also have no family history of ovarian or breast cancers; however, the populations mostly to be screened would include such individuals. Thus, it is unclear whether these two "control" populations would be identical. Despite these limitations, these results serve as a proof of concept that the analyses of specific microRNAs associated with circulating exosomes can be applied to all stages of ovarian cancer and that benign and malignant diseases appear distinguishable based on the levels of these 8 specific microRNAs.

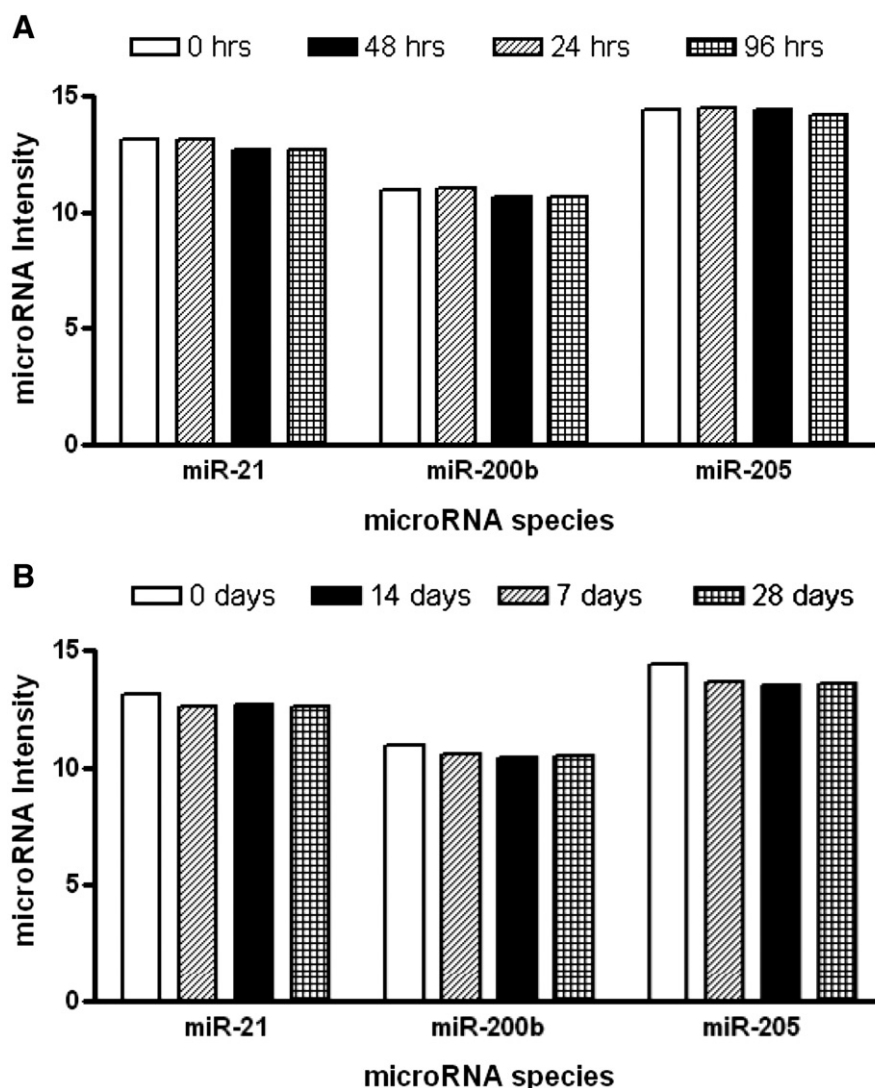


Fig. 5. Comparison of specific exosomal microRNAs derived from the serum of an ovarian cancer patient, immediately after blood draw or 24, 48, and 96 h later with sera stored at 4 °C (panel A) or after 7 to 28 days, stored at -70°C (panel B). Tumor exosomes were isolated by MACS using anti-EpCAM.

The microRNA signatures of exosomes parallel that of the microRNA-expression profiles of the originating tumor cells, indicating that microRNA profiling can be performed in the absence of tissue and accurately reflect the tumor's profile. We also have observed that tumor-derived exosomes from lung cancer patients contain microRNA that is similar to the corresponding tumor microRNA signatures. Circulating tumor-derived exosomes can be easily isolated using tumor markers, such as EpCAM, followed by analysis of exosome-associated microRNA. Since this approach is non-invasive, in that it does not require a mass to be biopsied, exosomal microRNA profiling has the potential to be used as a screening tool for the detection of cancer. As specific microRNAs associated with tumor tissues are identified that predict prognosis, including therapeutic resistance (such as let-7i, miR-16, miR-21 and miR-214) [31,32], their presence in tumor exosomes can also be assessed to further define the utility of exosomal microRNA profiling as a prognostic indicator. While validation studies will be necessary prior to

bypassing the use with tumor mass biopsies, the use of exosomal microRNA profiling could extend this approach to screening of asymptomatic individuals, as well as for monitoring disease recurrence.

Conflicts of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ygyno.2008.04.033](https://doi.org/10.1016/j.ygyno.2008.04.033).

References

- [1] Sankaranarayanan R, Ferlay J. Worldwide burden of gynaecological cancer: the size of the problem. *Best Pract Res Clin Obstet & Gynaecol* 2006;20:207–25.
- [2] Berek JS, Schultes BC, Nicodemus CF. Biologic and immunologic therapies for ovarian cancer. *J Clin Oncol* 2003;21(s10):168–74.
- [3] Menon U, Jacobs IJ. Recent developments in ovarian cancer screening. *Curr Opin Obstet Gynecol* 2000;12:39–42.
- [4] Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, et al. MicroRNA signatures in human ovarian cancer. *Cancer Res* 2007;67:8699–707.
- [5] De Cecco L, Marchionni L, Gariboldi M, Reid JF, Lagonigro MS, Caramuta S, et al. Gene expression profiling of advanced ovarian cancer: characterization of a molecular signature involving fibroblast growth factor 2. *Oncogene* 2004;23:8171–83.
- [6] Calin GA, Croce CM. MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res* 2006;66:7390–4.
- [7] Esquela-Kerscher A, Slack FJ. Oncomirs — microRNAs with a role in cancer. *Nature Rev Cancer* 2006;6:259–69.
- [8] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–97.
- [9] Miska EA. How microRNAs control cell division, differentiation, and death. *Curr Opin Genet Dev* 2005;5:563–8.
- [10] Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nature Rev Cancer* 2006;6:857–66.
- [11] Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834–8.
- [12] Zhang L, Huang J, Yang N, et al. microRNAs exhibit high frequency genomic alterations in human cancer. *Proc Natl Acad Sci USA* 2006;103:9136–41.
- [13] Taylor DD, Doellgast GJ. Quantitation of eroxidise-antibody binding to membrane fragments using column chromatography. *Anal Biochem* 1979;98:53–9.
- [14] Taylor DD, Homesley HD, Doellgast GJ. Binding of specific peroxidise-labeled antibody to placental-type alkaline phosphatase on tumor-derived membrane fragments. *Cancer Res* 1980;40:4964–9.
- [15] Raposo G, Tenza D, Mecheri S, Peronet R, Bonnerot C, Desaymard C. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Mol Biol Cell* 1997;8:2631–45.
- [16] Heijnen HFG, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ. Activation platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha granules. *Blood* 1999;94:3791–9.
- [17] Taylor DD, Black PH. Shedding of plasma membrane fragments: Neoplastic and developmental importance. In: Steinberg M, editor. *Developmental Biology*, vol. 3; 1986. p. 33–57.
- [18] Taylor DD, Bohler HC, Gercel-Taylor C. Pregnancy-linked suppression of TcR signaling pathways by a circulating factor absent in recurrent spontaneous pregnancy loss. *Molecular Immunology* 2006;43:1872–80.
- [19] Sabapatha A, Gercel-Taylor C, Taylor DD. Specific isolation of placental-derived exosomes from the circulation of pregnant women and their immunoregulatory consequences. *Am J Reprod Immunol* 2006;56:345–55.
- [20] Taylor DD, Gercel-Taylor C. Tumour-derived exosomes as mediates of T-cell signaling defects. *Brit J Cancer* 2005;92:305–11.
- [21] Olver C, Vidal M. Proteomic analysis of secreted exosomes. *Subcell Biochem* 2007;43:99–131.
- [22] Mears R, Craven RA, Hanrahan S, et al. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. *Proteomics* 2004;4:4019–31.
- [23] Bard MP, Hegmans JP, Hemmes A, et al. Proteomic analysis of exosomes isolated from human malignant pleural effusions. *Am J Respir Cell Mol Biol* 2004;31:114–21.
- [24] Choi DS, Lee JM, Park GW, et al. Proteomic analysis of microvesicles derived from human colorectal cancer cells. *J Proteome Res* 2007;6:4646–55.
- [25] Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem* 1998;273:20121–7.
- [26] Andre F, Scharz NE, Movassagh M, et al. Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* 2002;360:295–305.
- [27] Valenti R, Huber V, Filipazzi P, Pilla L, Sovena G, Villa A, et al. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer Res* 2006;66:9290–8.
- [28] Koga K, Matsumoto K, Akiyoshi T, Kubo M, et al. Purification, characterization and biological significance of tumor-derived exosomes. *Anticancer Res* 2005;25:3703–7.
- [29] Ratajczak J, Miekus K, Kucia M, et al. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 2006;20:847–56.
- [30] Valadi H, Ekstrom K, Bossius A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNA and microRNA is a novel mechanism of genetic exchange. *Nature Cell Biol* 2007;9:652–9.
- [31] Yang H, Kong W, He L, Zhao JJ, O'Donnell JD, Wang J, et al. MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. *Cancer Res* 2008;68:425–33.
- [32] Paul E, Blower PE, Chung JH, Verducci JS, Lin S, Park JK, et al. MicroRNAs modulate the chemosensitivity of tumor cells. *Mol Cancer Therap* 2008;7:1–9.