Exosome-derived miRNAs and ovarian carcinoma progression

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The objective of this study was to analyze the expression, biological role and clinical relevance of exosomal microRNAs (miRNAs) from ovarian carcinoma (OC) effusion supernatants. Exosomal miRNA expression profiling was performed using miRNA Taqman arrays. Selected miRNAs were validated using quantitative PCR in 86 OC effusion supernatants. The role of exosomal miRNA in this cancer was further studied using in vitro and in vivo models. miRNA profiling identified 99 miRNAs with high expression levels in exosomes from OC effusion supernatants. Quantitative PCR validation of 11 miRNAs showed significant associations with effusion site (peritoneum versus pleura) and International Federation of Gynecology and Obstetrics stage. In univariate survival analysis, high levels of miRNAs 21, 23b and 29a were associated with poor progression-free survival (P = 0.01, P = 0.015 and P = 0.009, respectively), whereas high expression of miRNA 21 correlated with poor overall survival (P = 0.017). The latter association was retained in Cox multivariate analysis (P = 0.001). Exposure of LP9 mesothelial cells and ES2 OC cells to OC effusion-derived exosomes inhibited tumor spheroid expansion and reduced mesothelial clearance area. Treatment of severe combined immunodeficiency mice with exosomes from OC effusions prior to injection of tumor cells was associated with larger tumor load, more infiltrative tumors and shorter survival. Patient-derived OC effusion exosomes contain multiple miRNAs, of which some may have clinical relevance. In experimental models, OC exosomes affect both tumor cells and cells in the tumor microenvironment and induce more aggressive disease. Collectively, these data demonstrate the central role of miRNAs and their content in the biology of this cancer.

Introduction

MicroRNAs (miRNAs, miRs), short non-coding RNAs, have a central role in regulation of gene expression in physiological and pathological conditions. miRNAs exert their regulatory function by binding to the 3’-untranslated regions of their target messenger RNA, thereby inhibiting target gene translation into proteins. Numerous studies using various profiling approaches have demonstrated deregulated miRNA profiles in various malignancies. Recent reports have identified circulating miRNAs, present in biological fluids such as blood, saliva, urine and ascites (1). These miRNAs have evident potential to serve as biomarkers for non-invasive diagnostic screening and early cancer detection (2). A few circulating miRNAs have been identified as tumor-specific and are regarded as promising cancer biomarkers (3,4).

Circulating miRNAs are released from donor cells to body fluids by two main pathways. The more studied one is through budding of membranes and creation of small membrane vesicles called exosomes that are secreted from the cells. Exosomes containing miRNA enable the latter to exist and travel in plasma and other fluids. The second, recently discovered pathway is by conjugation of miRNAs with RNA-binding proteins, such as Ago2. The complex is released from the cell and circulates in body fluids (5). Both pathways assure the stability of circulating miRNAs by providing protection from degradation.

Exosomes are 30–100 nm lipoprotein vesicles that are secreted from cells and present in most circulating body fluids. Exosomes contain proteins, messenger RNAs and miRNAs (6–8). Although the protein composition of exosomes is largely unvarying, the RNA content depends on the origin of the exosomes. The mechanisms of sorting and packaging of RNA into exosomes are not well-defined, but it is understood that this process can be selective, favoring certain RNAs for exosomal cargo over others (9).

Ovarian carcinoma (OC) is the most lethal gynecological cancer, ranking fifth in cancer-related deaths among women in the Western world (10). In a recent study, we characterized the differences in miRNA expression pattern between primary tumors versus effusion-derived tumor cells in OC (11). In the present study, we extended our evaluation to the miRNA population found in the effusion fluid supernatant of OC patients. Our results indicate on selective sorting of certain miRNAs into the exosomal fractions and provide evidence that the exosome profile is related to disease outcome.

Materials and Methods

Patients and material

Specimens and clinical data were obtained from the Department of Gynecologic Oncology, Norwegian Radium Hospital. Informed consent was obtained according to national Norwegian and international guidelines. Study approval was given by the Regional Committee for Medical Research Ethics in Norway.

Effusions. Effusions were submitted for routine diagnostic purposes during 1998–2003 and were processed immediately after tapping. Fresh non-fixed malignant peritoneal (n = 66) and pleural (n = 20) effusions were obtained from 86 patients, including 71 with OC, 10 with primary peritoneal carcinoma and 5 with tubal carcinoma. Due to their closely linked histogenesis and phenotype, these tumors are all referred to as OC henceforth.

Forty-seven effusions were obtained prior to chemotherapy administration, and 39 were obtained post-chemotherapy, the majority at recurrence, disease. The majority of patients (82/86; 88%) received platinum-based therapy. Clinicopathologic data are detailed in Table I. Grading was according to the International Federation of Gynecology and Obstetrics (FIGO) system. All serous OC were high-grade serous carcinomas (HGSC).

Effusion specimens were centrifuged immediately after tapping, and cell supernatants were frozen at –70°C with no additional reagents. Conventional Papanicolaou (Pap)- and Diff-Quick–stained smears and cell blocks were prepared and diagnoses were established using morphology and immunohistochemistry. Cell pellets from 13 OC effusions previously analyzed for miRNA profile (11) and supernatants from eight benign effusions containing a large population of reactive mesothelial cells (RMC) served as reference controls.

Cell lines

The ES2 OC cell line was cultured in the appropriate media (Biological Industries, Beit-Haemek, Israel) according to the manufacturer’s instructions. LP9, a mesothelial cell line generated from the benign mesothelium of an OC patient, was cultured in 1:1 mixture of Ham’s F12 medium and Medium 199 with 10% fetal calf serum (Biological Industries). The medium was supplemented with 1 ng/ml insulin (Sigma–Aldrich, St Louis, MO), 100 μg/ml ascorbic acid (Sigma–Aldrich), 100 U/ml of penicillin–streptomycin–glutamine (Sigma–Aldrich) and 2 mM L-glutamine (Biological Industries). All cells were grown in a humidified atmosphere of 95% air and 5% CO₂.

Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; HGSC, high-grade serous carcinomas; miRNA, microRNA; OC, ovarian carcinoma; OS, overall survival; PFS, progression-free survival; RMC, reactive mesothelial cells.

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Serous carcinomas were all HGSC; non-serous carcinomas included two mucinous, three clear cell, two endometrioid and two mixed-type carcinomas.

One patient with ovarian carcinoma operated at another hospital for whom the primary tumor could not be accessed for evaluation.

Including patients who were not operated, patients who received neoadjuvant chemotherapy, and patients for whom the primary tumor could not be accessed for evaluation of grade.

NA, not available; including with allergic response or adverse effects of chemotherapy and patients for whom chemotherapy response could not be evaluated because of normalized CA-125 after primary surgery or missing CA-125 information.

The ES2 cells were purchased from American Type Culture Collection 4 years ago. The LP9 cells were obtained from Coriell Cell Repositories (Camden, NJ) 4 years ago. There is at present no facility to test cell lines in Israel, although a process to establish such a center is well under-way at the Technion University in Haifa. We have sent the cells for validation there, but are forced to wait until this process is completed. The cell lines are kept and used under rigorous conditions at the laboratory of Professor Reich.

Exosome precipitation

Two hundred fifty microliters of cell-free effusion supernatant was mixed with 63 μl of ExoQuick (SBI, Mountain View, CA), followed by incubation and centrifugations according to the manufacturer’s instructions. Exosome pellets were obtained and lysed using 300 μl of miRvana lysis buffer (Ambion, Austin, TX), followed by RNA extraction using the miRvana miRNA Isolation Kit (Ambion). RNA quantity and quality were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA).

miRNA TaqMan arrays

One thousand nanograms of total RNA were pooled for OC cells, OC effusion supernatants and RMC supernatants. RNA was concentrated and purified by ethanol precipitation. RNA pellet was resuspended in 3 μl of DEPC water and the Megaplex Pools protocol (without preamplification) with Megaplex RT Human Pool A Primers (Applied Biosystems, Foster City, CA) was followed. The obtained RT products were used for TaqMan MicroRNA Arrays A (Applied Biosystems) according to the manufacturer’s instructions. Arrays were performed on ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). Data were analyzed with SDS Relative Quantitation software (Applied Biosystems) using U6 as reference gene.

Target and pathway prediction

Putative targets for miRNAs of interest were predicted using the TargetScan (http://www.targetscan.org) algorithm. Gene annotations and specific pathways were fingerprinted out using online free access Pathway-Express software (Intelligent Systems and Bioinformatics Laboratory, Computer Science Department, Wayne State University, Detroit, MI).

Quantitative real-time PCR

Eighty nanograms of total RNA were converted into cDNA and quantitative PCR detection of miRNAs was carried out according to the manufacturer’s protocols using the miScript Reverse Transcription Kit and miScript SYBR Green PCR Kit (Qiagen GmbH, Hilden, Germany). Optimized miRNA-specific primers for each miRNA, as well as for the endogenous control 5S, were commercially available (miScript Primer Assays, Qiagen GmbH). All experiments were performed in duplicate and PCR specificity was confirmed by appropriate melting curves.

Exosomes for in vitro and in vivo studies

Exosome pellets were obtained as previously described and resuspended in 200 μl of phosphate-buffered saline. Protein content was evaluated by the Bradford assay. The re-suspended exosomes were pooled together according to the protein content measurements. Twenty micrograms of exosomes were used for each of the different in vitro assays, and 50 μg of exosomes were injected intra-peritoneally three times to severe combined immunodeficiency mice for the in vivo assay.

Spheroid-induced mesothelial clearance assay in the presence of exosomes

Mesothelial clearance assay was based on a method described by Iwanicki et al. (12).

Mesothelial cells (LP9) were labeled with PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma–Aldrich), plated on a 24-well tissue culture plate and maintained in medium without fetal calf serum, supplemented with bovine serum albumin (Sigma–Aldrich), until confluent (48 h after plating). After labeling and plating, mesothelial cells were exposed to 20 μg of exosomes for a period of 48 h. The results were compared to naive mesothelial cells.

To generate spheroids, ES2 cells were dissociated by trypsin solution, re-suspended and placed in 60 mm tissue culture dishes for agitation in order to prevent cell attachment to the culture plate and to facilitate spheroid formation. The medium contained 20 μg of exosomes (extracted from patient samples, as previously described). Spheroids were collected for experiments 48 h later.

In co-culture experiments, spheroids were added to a confluent mesothelial monolayer that was either naive or previously exposed to 20 μg of exosomes, allowed to attach and imaged at 3, 24 and 48 h with Olympus FV300 laser scanning confocal system, using Olympus IX70 microscope (Olympus, Center Valley, PA). Quantification of both the spheroid and the clearance areas was assessed using Olympus digital imaging software.

miRNA plasmids for stable overexpression

miExpress precursor miRNA expression vectors were used for stable overexpression of miR-21 (GC-HmiR0284-MR04-B) and miR-29a (GC-HmiR0119-MR04-B), respectively (GeneCopoeia, Rockville, MD). For transfection, 5 × 106 ES-2 cells were plated in 24-well plates 24 h prior to transfection and were treated with 350 ng of the obtained plasmid DNAs using Lipofectamine Reagent. Transfected cells were selected using 0.8 μg/ml puromycin (AG Scientific, San Diego, CA). Transfection efficiency was assessed by quantitative real-time PCR.

OC mouse model in the presence of exosomes

For generation of an OC mouse model, 5- to 6-week-old severe combined immunodeficiency mice were used. Fifty micrograms of isolated exosomes were injected into the peritoneum of mice (n = 8). Injections were repeated three times on alternate days. Forty-eight hours following the final injection of exosomes, mice were injected with 5 × 105 ES2 cells directly into each ovary. Following injection, these cells produced local and distant tumors and effusions. Mice were observed daily and sacrificed 14 days after injection. Control mice (n = 8) were injected with RMC-generated exosomes prior to tumor injection. Tumors were removed and fixed in 4% formaldehyde and embedded in paraffin, and 4 μm sections were stained with hematoxylin and eosin. The joint ethics committee of the Hebrew University and Hadassah Medical Center approved the study protocol for animal welfare.

Statistical analysis

Statistical analysis was performed applying the SPSS-PC package (Version 18.0, Chicago, IL). Probability of <0.05 was considered statistically significant. Analysis of the association between miRNA expression in effusion exosomes and clinicopathologic parameters was undertaken using the Mann–Whitney U-test. Clinopathologic parameters were grouped as follows: Age: ≤60 versus >60 years; effusion site: peritoneal versus pleural; FIGO stage: III versus IV; chemotherapy status: pre- versus post-chemotherapy; residual disease volume: ≤1 cm versus >1 cm; response to chemotherapy for primary disease: complete versus partial response/stable disease/progression. Chemotherapy response was defined according to RECIST criteria (13,14).

Progression-free survival (PFS) and overall survival (OS) were calculated from the date of the last chemotherapy treatment/diagnosis to the date of recurrence/death or last follow-up, respectively. Univariate survival analyses of PFS

Table I. Clinicopathologic data of the effusion cohort (86 patients)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, range</td>
<td>63; 35–83</td>
</tr>
<tr>
<td>FIGO stage</td>
<td>III: 45</td>
</tr>
<tr>
<td></td>
<td>IV: 41</td>
</tr>
<tr>
<td>Histology&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Serous: 76</td>
</tr>
<tr>
<td></td>
<td>Non-serous: 9</td>
</tr>
<tr>
<td>Grade</td>
<td>1: 8</td>
</tr>
<tr>
<td></td>
<td>2: 16</td>
</tr>
<tr>
<td></td>
<td>3: 50</td>
</tr>
<tr>
<td>Residual disease</td>
<td>≤1 cm: 23</td>
</tr>
<tr>
<td></td>
<td>&gt;1 cm: 46</td>
</tr>
<tr>
<td>Chemotherapy response at diagnosis</td>
<td>Complete: 43</td>
</tr>
<tr>
<td></td>
<td>Partial response: 12</td>
</tr>
<tr>
<td></td>
<td>Stable disease: 2</td>
</tr>
<tr>
<td></td>
<td>Progressive disease: 20</td>
</tr>
<tr>
<td></td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;: 9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Serous carcinomas were all HGSC; non-serous carcinomas included two mucinous, three clear cell, two endometrioid and two mixed-type carcinomas.

<sup>b</sup>One patient with ovarian carcinoma operated at another hospital for whom the primary tumor could not be accessed for evaluation.

<sup>c</sup>Including patients who were not operated, patients who received neoadjuvant chemotherapy, and patients for whom the primary tumor could not be accessed for evaluation of grade.

<sup>d</sup>NA, not available; including with allergic response or adverse effects of chemotherapy and patients for whom chemotherapy response could not be evaluated because of normalized CA-125 after primary surgery or missing CA-125 information.
and OS were executed using the Kaplan–Meier method and log-rank test. Expression categories in survival analyses were clustered as high versus low based on messenger RNA and protein median expression level. Multivariate analyses of OS and PFS were performed using the Cox proportional hazard model (Enter function).

Differences among groups in the in vitro and in vivo assays were calculated using the Mann–Whitney U- and Kruskal–Wallis H-tests. The Mann–Whitney U-test was applied to comparative analysis of the diameter and the depth of infiltration of intra-abdominal metastases (added value for both) in OC exosome-treated versus RMC exosome-treated mice.

**Results**

**miRNA signatures of effusion-derived exosomes**

We pooled together exosomes derived from OC effusion supernatants (n = 9) and used TaqMan miRNA array platforms for the analyses. Exosomes derived from pooled RMC effusions (n = 8) served as a benign control. We also used an array of pooled (n = 13) effusion-derived tumor cell miRNA as reference.

Following analysis, highly expressed miRNAs (Ct < 30 cycles) were detected. We identified 99 miRNAs with high expression levels in the exosomes from OC effusion supernatants. We compared these miRNAs with 102 miRNAs that were highly expressed in OC cells in effusion specimens and with 14 miRNAs that were highly expressed in RMC. Our results indicate that about 75% of the miRNAs in the effusion supernatant may be have origin in OC cells, with lesser contribution by RMC (Figure 1).

Next, we attempted to find the possible origin of the remaining 23 miRNAs by comparing them with a previously published list of miRNAs present in normal female serum (15). Serum could be identified as the likely source of only 4/23 miRNAs, leaving 19 effusion supernatant-specific miRNAs (Table II) with yet unknown origin and role.

In order to understand the possible role of those extracellular miRNAs, we looked for a common pathway they might control. We used TargetScan to identify top 20 predicted targets for each miRNA. Those predicted targets were then combined and processed with Pathway-Express software. Pathways with the highest impact factors, and thereby most likely to be affected by the miRNAs of interest, are listed in Table III.

**Table II. miRs uniquely overexpressed in effusion supernatants**

<table>
<thead>
<tr>
<th>miR</th>
<th>Impact factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-127-3p</td>
<td>10.632</td>
</tr>
<tr>
<td>hsa-miR-129-3p</td>
<td>9.095</td>
</tr>
<tr>
<td>hsa-miR-133a</td>
<td>8.342</td>
</tr>
<tr>
<td>hsa-miR-133b</td>
<td>7.365</td>
</tr>
<tr>
<td>hsa-miR-134</td>
<td>6.886</td>
</tr>
<tr>
<td>hsa-miR-140-5p</td>
<td>6.075</td>
</tr>
<tr>
<td>hsa-miR-145</td>
<td>5.524</td>
</tr>
<tr>
<td>hsa-miR-152</td>
<td>10.632</td>
</tr>
<tr>
<td>hsa-miR-219-5p</td>
<td>9.095</td>
</tr>
<tr>
<td>hsa-miR-323-3p</td>
<td>8.342</td>
</tr>
<tr>
<td>hsa-miR-328</td>
<td>7.365</td>
</tr>
<tr>
<td>hsa-miR-346</td>
<td>6.886</td>
</tr>
<tr>
<td>hsa-miR-365</td>
<td>6.424</td>
</tr>
<tr>
<td>hsa-miR-376c</td>
<td>5.573</td>
</tr>
<tr>
<td>hsa-miR-422a</td>
<td>5.524</td>
</tr>
<tr>
<td>hsa-miR-452</td>
<td>5.456</td>
</tr>
<tr>
<td>hsa-miR-532-3p</td>
<td>5.456</td>
</tr>
<tr>
<td>hsa-miR-598</td>
<td>5.456</td>
</tr>
<tr>
<td>hsa-miR-95</td>
<td>5.456</td>
</tr>
</tbody>
</table>

ECM, extracellular matrix; TGFβ, transforming growth factor beta.

**Fig. 1.** Origin of exosomal miRNA in OC effusions. Venn diagram presentation of miRNAs in effusions: miRNAs highly expressed in effusion supernatant-derived exosomes in yellow, miRNAs highly expressed in effusion-derived tumor cells in blue, miRNAs highly expressed in exosomes derived from reactive mesothelium in green.

**Exosomal miRNA levels are associated with clinicopathologic parameters and survival in OC**

The levels of 11 of the 99 miRNAs with high expression levels in the exosomes were analyzed for association with clinicopathologic parameters in a series of 86 effusions. Significant associations related to effusion site and FIGO stage are presented in Supplementary Table 1, available at Carcinogenesis Online. No significant associations were seen with chemotherapy exposure, patient age, histological grade, residual disease volume, or response to chemotherapy at diagnosis (P > 0.05).

In analysis limited to HGSC (n = 76), significant associations were seen with respect to effusion site, FIGO stage and residual disease volume (Supplementary Table 1, available at Carcinogenesis Online).

Median PFS for all patients (n = 86) was 4 months (range 0–82) and OS was 23 months (range 1–110). At the last follow-up, two patients had no evidence of disease, two were alive with disease and 82 were dead of disease.

In univariate survival analysis, high expression of miRNA 21 correlated with poor OS (P = 0.017; Figure 2A). The clinicopathologic parameters significantly related to OS were histological grade (P = 0.016) and residual disease volume (P = 0.03), with a trend for patient age (P = 0.142). FIGO stage was unrelated to OS (P = 0.844). In multivariate survival analysis, miRNA 21 (P = 0.001) and age (P = 0.002) were independently associated with poor OS.

In univariate survival analysis of PFS, high levels of miRNAs 21, 23b and 29a were associated with poor PFS (P = 0.01, P = 0.015 and P = 0.009, respectively; Figure 2B–D). Residual disease volume was the only clinicopathologic parameter related to PFS (P = 0.013). None of these parameters was independent prognosticator in multivariate Cox survival analysis (data not shown).
The association between high expression of miRNA 21 and poor OS ($P = 0.044$), and between high levels of miRNAs 21 and 29a and poor PFS ($P = 0.023$ and $P = 0.006$, respectively) was retained in survival analysis limited to HGSC (data not shown).

In univariate survival analysis of pre-chemotherapy effusions obtained at diagnosis ($n = 47$), high levels of miRNAs 21 and 29a were associated with poor OS ($P = 0.018$ and $P = 0.011$, respectively) and poor PFS ($P = 0.007$ and $P = 0.001$; Figure 2E–H).

Effects of exosomes on the metastatic potential in vitro and in vivo

In an attempt to elucidate the messenger effect of exosomes in OC, we tested the effects of patient-derived exosomes with a mesothelial clearance assay (12). Spheroids were generated from ES2 OC cells either alone or in the presence of pooled exosomes from OC patients. LP9 mesothelial cells were naive or were also exposed to the exosome treatment. The three experiment groups were as follows: Group A: spheroids were generated in the presence of exosomes, mesothelial cells were naive or were also exposed to the exosome treatment. The three experiment groups were as follows: Group A: spheroids were generated in the presence of exosomes, mesothelial cells were naive or were also exposed to the exosome treatment. Group B: both spheroids and mesothelial cells were treated with exosomes; Group C: naive spheroids and exosome-treated mesothelial monolayer. We then measured spheroid size and the area of induced mesothelial clearance at 3, 24 and 48 h.

There was no effect of OC-derived exosomes on spheroid size or mesothelial clearance when tumor spheroids alone were treated with exosomes (Group A) compared to controls. However, exposure of mesothelial cells to OC-derived exosomes altered the behavior of both spheroid and mesothelial cells, evidenced by inhibited spheroid expansion and reduced mesothelial clearance area, both seen at 24 and 48 h. These results were most significant for group C, with similar effect, though of lesser extent, seen for group B (Figure 3). These findings suggest that the exosomal effect is mostly exerted on mesothelial cells, i.e. the tumor microenvironment within the peritoneal and pleural body cavities, rather than on the tumor itself.

In order to further understand the effects of OC-derived exosomes in vivo, we pre-treated severe combined immunodeficiency mice with isolated exosomes prior to injection of cancer cells. Fifty micrograms of pooled exosomes isolated from effusions of OC patients were injected into the peritoneum of mice. The injections were repeated three times at alternate days. Mice were injected with ES2 cells 48 h following the final injection of exosomes. Mice injected with exosomes prior to ES2 cells had more aggressive disease with significantly higher tumor load ($P = 0.022$; Supplementary Table 2, available at Carcinogenesis Online), more infiltrative tumors ($P = 0.011$; Supplementary Table 2, available at Carcinogenesis Online) and shorter OS (Figure 4) compared to controls.

miR-21 and miR-29a modulate mesothelial clearance in vitro

Since exosomes contain a variety of components in addition to miRNAs, we examined the specific effect of two miRNAs, miR-21 and miR-29a, which were shown to correlate with poor OS and shorter PFS, respectively, in our cohort. Stable transfection of these specific miRs into OC cells resulted in increased clearance area in our mesothelial clearance assay described above (Supplementary Figure 1, available at Carcinogenesis Online), supporting our hypothesis that the enlarged mesothelial clearance area observed following exposure to exosomes is mediated by miRNAs in tumor cells.
Exosomes in ovarian carcinoma

Discussion

It is currently recognized that exosomes are not merely cellular debris, but a means of communication between cells. They can shuttle their content from one cell to another by three main mechanisms: receptor-mediated uptake, direct fusion with the plasma membrane or endocytosis by phagocytosis (16). Irrespective of the mechanism, exosomes are taken up by distant cells and their content influences the recipient. The first example of such cell–cell communication using exosomal miRNA was demonstrated by Valadi et al. (7).

Fig. 2. Higher exosomal miRNA levels are associated with poor survival in OC. (A) Kaplan–Meier survival curve showing the association between mir-21 levels in exosomes from OC effusion supernatants and OS for 86 patients. Patients with effusions showing low (below median) mir-21 expression (n = 43, solid line) had a mean OS of 36 months compared to 23 months for patients with effusions with high mir-21 expression (n = 43, dashed line; P = 0.017). (B) Kaplan–Meier survival curve showing the association between mir-21 levels in exosomes from OC effusion supernatants and PFS for 86 patients. Patients with effusions showing low (below median) mir-21 expression (n = 43, solid line) had a mean PFS of 12 months compared to 5 months for patients with effusions with high mir-21 expression (n = 43, dashed line; P = 0.01). (C) Kaplan–Meier survival curve showing the association between mir-23b levels in exosomes from OC effusion supernatants and PFS for 86 patients. Patients with effusions showing low (below median) mir-23b expression (n = 43, solid line) had a mean PFS of 13 months compared to 5 months for patients with effusions with high mir-23b expression (n = 43, dashed line; P = 0.015). (D) Kaplan–Meier survival curve showing the association between mir-29a levels in exosomes from OC effusion supernatants and PFS for 86 patients. Patients with effusions showing low (below median) mir-29a expression (n = 43, solid line) had a mean PFS of 12 months compared to 5 months for patients with effusions with high mir-29a expression (n = 43, dashed line; P = 0.009). (E) Kaplan–Meier survival curve showing the association between mir-21 levels in exosomes from OC effusion supernatants and OS for 47 patients with pre-chemotherapy effusions. Patients with effusions showing low (below median) mir-21 expression (n = 23, solid line) had a mean OS of 39 months compared to 20 months for patients with effusions with high mir-21 expression (n = 24, dashed line; P = 0.018). (F) Kaplan–Meier survival curve showing the association between mir-21 levels in exosomes from OC effusion supernatants and OS for 47 patients with pre-chemotherapy effusions. Patients with effusions showing low (below median) mir-21 expression (n = 23, solid line) had a mean OS of 39 months compared to 20 months for patients with effusions with high mir-21 expression (n = 24, dashed line; P = 0.018). (G) Kaplan–Meier survival curve showing the association between mir-29a levels in exosomes from OC effusion supernatants and OS for 47 patients with pre-chemotherapy effusions. Patients with effusions showing low (below median) mir-29a expression (n = 23, solid line) had a mean OS of 39 months compared to 20 months for patients with effusions with high mir-29a expression (n = 24, dashed line; P = 0.007). (H) Kaplan–Meier survival curve showing the association between mir-29a levels in exosomes from OC effusion supernatants and PFS for 47 patients with pre-chemotherapy effusions. Patients with effusions showing low (below median) mir-29a expression (n = 23, solid line) had a mean OS of 19 months compared to 3 months for patients with effusions with high mir-29a expression (n = 24, dashed line; P = 0.001).

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who showed that addition of exosomes derived from mouse mast cells to human mast cells resulted in detection of mouse proteins in human cells. In a similar fashion, exosomal miRNAs are transferred into the recipient cell and influence protein expression and cellular phenotype.

Two aspects of exosomes are of obvious relevance in cancer. First, tumor-derived exosomes may comprise RNA signatures that are organ-specific. Circulating exosomal miRNAs can serve as biomarkers to improve disease diagnosis by distinguishing healthy from malignant individuals. Taylor et al. (17) profiled miRNAs of exosomes isolated from serum of OC patients and found specific miRNA signatures that can be used in screening for early diagnosis in this cancer.

The second aspect is the ability of tumor-derived exosomes to deliver messages from one cell to another. There are several studies that suggest that tumor-derived exosomes can educate other cells to aid tumor survival and promote metastasis. Prostate cancer cells evade the immune system by inducing apoptosis of cytotoxic T-lymphocytes via exosomes (18), whereas melanoma-derived exosomes promote angiogenesis by affecting endothelial cells (19) and educate bone marrow cells to obtain metastatic properties (20). Hood et al. (19) showed that melanoma-derived exosomes facilitate lymphatic metastasis, by preparing pre-metastatic niches at lymph nodes. Using exosomes in a paracrine fashion, tumor cells influence both their microenvironment and distant tissues. This exosome-mediated reprogramming of normal cells allows increased proliferation, migration and metastasis of cancer cells.

The present study focused on miRNAs found in exosomes present in OC effusion supernatants, with the aim of profiling exosomal miRNA expression, and analyzing their prognostic role. We then tested the effects of isolated exosomes on OC progression in vitro and in vivo.

The ES-2 line used in the present study is derived from a clear cell OC, whereas the majority of clinical specimens studied were HGSC. The reason for using this cell line was the fact that it lends itself easier than other OC cell lines we have assessed in vitro and in vivo to transfection, including that of miRNAs. Although the molecular profile of clear cell OC differs from that of its serous counterpart, the ability of the ES-2 line to generate multifocal peritoneal disease in the same manner HGSC do may justify its use as a model for carcinoma–mesothelium interactions and disease progression in this disease.

miRNAs are growingly perceived to have a major role in regulating the expression of cancer-associated molecules in OC, and their expression has been shown to be related to chemotherapy response, disease progression and survival in this cancer [reviewed in (21)]. In the present study, we identified significant associations between the levels of miR-21, miR-23a, miR-23b, miR-29a, miR-99a, miR-125b, miR-200c, miR-320a and miR-484 and clinicopathologic parameters and/or survival in our cohort, of which miRNAs 21, 23b and 29a were associated with poor survival. Three of these miRs, i.e. miR-21, miR-29a and miR-200c were previously reported to be overexpressed in serum from OC patients compared to controls, suggesting a potential role in OC diagnosis (22,23).

miR-21 negatively regulates the expression of the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) in OC, resulting in proliferation, invasion, migration and hypoxia resistance (24,25). We found expression of miR-21 in OC cells (11) and in effusion fluid-derived exosomes, and the latter was an independent prognostic marker of poor OS. The high expression of miR-21 in metastatic OC may be one of the mechanisms mediating chemoresistance in this cancer.

**Fig. 3.** Effect of patient-derived OC exosomes on mesothelial clearance in vitro. Spheroid size and the area of induced mesothelial clearance were measured at 3, 24 and 48 h. (A) spheroids were generated in the presence of exosomes, mesothelial cells were exosome-naive; (B) both spheroids and mesothelial cells were treated with exosomes; (C) exosome-naive spheroids and exosome-treated mesothelial monolayer.

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... to low expression of PTEN, a validated target of miR-23b, which is observed in OC cells in effusions (28). The data by other groups and us suggest a tumor-promoting role for miR-23a and miR-23b in OC.

The mir-29 family was recently reported to be aberrantly expressed in multiple cancers including HGSC (29) and targets numerous cancer pathways, mediating deregulation of apoptosis, cell cycle induction and metastasis formation. Higher miR-29b levels were previously reported to be associated with poor disease-free survival in OC (30). Our previous (11) and current data show that miR-29a is expressed in OC cells and in secreted OC exosomes and that its overexpression is significantly related to shorter PFS. The data of other investigators and us suggest a tumor-promoting role for miRs 29a and 29b in OC.

Data regarding the other above-listed miRNAs, including miR-125b, the miR-200 family and miR-484, have been recently reviewed (21).

Recent studies demonstrated that pre-treatment of experimental mice with tumor-derived exosomes increases tumor burden and decreases survival (19). It was suggested that exosomes may prepare the potential tumor niche in a way that turns it into a more favorable environment for homing tumor cells. Our experiments conducted with mesothelial cells show that exposure of the mesothelial cell layer to effusion fluid-derived exosomes increases mesothelial clearance, which may simulate modulation of tumor cell invasion in the peritoneum of OC patients. Exosomes may thus transfer certain regulatory elements or information into the mesothelial cells, which accelerate retraction and exposure of the sub-mesothelial matrix to invasion by tumor cell spheroids. In agreement with this hypothesis, pre-treatment of experimental mice with exosomes resulted in larger tumor burden and shorter survival.

Our data suggest that OC cells specifically direct certain miRNAs into exosomes that are secreted into the effusion fluid. These exosomes in turn educate the mesothelial cell layer to allow tumor spheroid dissemination through the peritoneal cavity. To our best knowledge, this is the first demonstration of such exosomal potential in OC.

Supplementary material

Supplementary Tables 1 and 2 and Figure 1 can be found at http://carcin.oxfordjournals.org/

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References