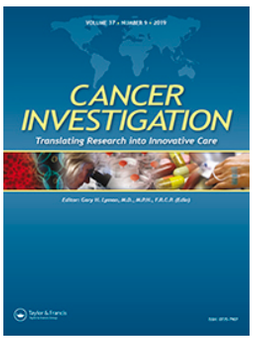

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Ovarian Cancer: Differentially Expressed microRNAs in Tumor Tissue and Cell-Free Ascitic Fluid as Potential Novel Biomarkers

Luděk Záveský[a], Eva Jandáková[b], Vít Weinberger[c], Luboš Minář[c], Veronika Hanzíková[d], Daniela Dušková[d], Lenka Záveská Drábková[e] and Aleš Hořínek[a,f]

[a]Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague 2, Czech Republic; [b]Department of Pathology, Masaryk University and University Hospital Brno, Brno, Czech Republic; [c]Department of Obstetrics and Gynecology, Masaryk University and University Hospital Brno, Brno, Czech Republic; [d]Faculty Transfusion Center, General University Hospital in Prague, Prague 2, Czech Republic; [e]Institute of Experimental Botany, Czech Academy of Sciences, Prague 6, Czech Republic; [f]3rd Department of Medicine, Department of Endocrinology and Metabolism, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague 2, Czech Republic

ABSTRACT

Ovarian cancer is the deadliest gynecologic cancer. The large-scale microRNA (miRNA) expression profiling and individual miRNA validation was performed to find potential novel biomarkers for ovarian cancer. The most consistent overexpression of miRs-200b-3p, 135 b-5p and 182-5p was found in both ascitic fluid and tumors and suggests their potential as oncogenes. miR-451a was consistently underexpressed so may be a tumor suppressor. Results were inconsistent for miR-204-5p, which was overexpressed in ascitic fluid but underexpressed in tumor tissue. miR-203a-3p was generally overexpressed but this failed to be proved in independent sample set in tissue validation.

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Ovarian cancer; ascites; effusion; microRNA; tumor

Introduction

Much effort in cancer research has been devoted to identify markers for diagnosis, indicating the development of cancer, its progression or predictive and prognostic links associated with responses to therapy or outcomes for patients. Most cases of ovarian cancer, the deadliest of gynecologic cancers (1), are found as advanced disease, restricting treatment options and exhibiting high levels of recurrence, eventually resulting in poor outcomes for patients (2). Screening and diagnostic options for ovarian cancer still remain inadequate (3,4).

Researchers have been exploring the group of non-coding RNAs known as microRNAs (miRNAs) for more than a decade (5) for developing more sensitive and specific methods of detecting cancer. Extensive data are also available for ovarian cancer, but we are still far from the routine use of miRNAs as diagnostic, prognostic or treatment tools in ovarian cancer (6,7).

Tumor tissues, cell lines, whole blood and plasma/serum have particularly been thoroughly investigated to identify suitable miRNA biomarkers for ovarian cancer (reviewed in (8,9)). Only three reports for cell-free urine miRNAs have yet been published for ovarian cancer (10–12).

Importantly, the spread of ovarian cancer into the peritoneal cavity may be mediated and promoted by the excessive accumulation of fluid known as ascites (effusion). Ascitic fluid is often associated with the development of ovarian cancer and poor prognoses for patients (13,14). In addition to cell components such as tumor cells, lymphocytes, mesothelial cells or macrophages, ascitic fluid contains diverse types of molecules, including soluble angiogenic and growth factors, cytokines, chemokines and extracellular matrix components that may contribute to cell growth, tumor invasion and resistance to TNF-related apoptosis-inducing ligands. The acellular fraction,

CONTACT Luděk Záveský ludek.zavesky@vfn.cz Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University and General University Hospital, Albertov 4, 128 00, Prague 2, Czech Republic

Supplemental data for this article can be accessed here.

however, may contain anti-angiogenic and apoptosis-promoting factors (15,16).

The specific biological roles of ascites, particularly those linked to ovarian carcinogenesis, remain to be elucidated in detail. Malignant ascites presents a considerable clinical challenge, but this bodily fluid also provides a wealth of opportunities for translational research (16) as the source of novel biomarkers.

Several recent studies have focused on ascites-derived miRNAs in cancer cells and on extracellular miRNAs or exosomal miRNAs in ascitic fluid (17–21). Large-scale comprehensive profiling and validation of expressed miRNA in cell-free fractions of ascitic fluid and comparing expression with tumor tissues, however, have been lacking. Our recent comprehensive study of miRNAs derived from cell-free ascitic fluid suggested their promising potential for use as novel biomarkers of ovarian cancer (22). In the present follow-up study, we explored miRNA expression in three types of biopsies associated with ovarian cancer: tumor tissue, cell-free ascitic fluid and urine. Large-scale profiling of miRNA expression and extensive validation was applied using ovarian carcinoma tissues. Individual candidate miRNAs were evaluated in all sample types based on the results and previously obtained data for ascites. Similar pattern of differential miRNA expression across different clinical samples may provide the evidence of their close linkage within ovarian carcinogenesis. Eventually, potential targets for evaluation of oncogene and tumor suppressor functions within regulatory miRNA networks can be tested for development of novel clinical biomarkers and therapy options in ovarian cancer.

Material and methods

Patients

This study was carried out in compliance with the Helsinki Declaration and was approved by the multi-centric Ethics Committee of the General University Hospital in Prague (VFN Praha). Ovarian-cancer patients provided samples of tumor tissue (Tissue experiment), ascitic fluid (Ascites experiment) or urine (Urine experiment), and patients with other gynecological disorders (normal ovaries) provided control samples (Tissue experiment). All patients were treated at the University Hospital in Brno (FN Brno). Healthy postmenopausal women provided blood samples for plasma isolation (Ascites experiment) or urine samples (Urine experiment) as negative controls, both at the Faculty Transfusion Center (VFN Praha). The plasma was used as an appropriate control for ascites, because it is the only bodily fluid associated with ascites and is not affected by any disease. All patients were Caucasians and provided written informed consent. The clinicopathological characteristics of the patients are summarized in Table 1 (Tissue

Table 1. Clinicopathological characteristics of the patients (Tissue experiment).

Diagnosis/type	Patients (%)
Primary serous ovarian cancer (OC2-14)	13 (93)
Primary serous/mucinous ovarian cancer (bilateral) (OC1)[a]	1 (7)
Control ovarian tissue (ON1-14)	14 (100)
Experimental screening: OC1-7 (cancer samples), ON1-7 (control samples).	7 (cancer), 7 (control)
Experimental validation: OC1-14 (cancer), ON1-14 (control)	14 (cancer), 14 (control)
Parameters (tumor tissue samples)	
Primary ovarian serous carcinoma	14 (100)
Grade	
High: OC2-9, 12, 13	10 (71)
Low: OC1, 10, 11, 14	4 (29)
FIGO stage	
FIGO I/II: Ia, OC10; Ic, OC7, 11, 14; IIb, OC2; IIc, OC8, 9, 13	8 (57)
FIGO III/IV: IIIb, OC1, 3, 4; IIIc, OC5, 6, 12	6 (43)
Median age	Years (range)
Cancer samples (ovarian cancer tissue)	66 (47–78)
Control samples (normal ovary tissue)	64.5 (46–78)

FIGO: International Federation of Gynecology and Obstetrics.
[a]Serous carcinoma sample was analyzed in the experiment.

Table 2. Clinicopathological characteristics of the patients (Ascites experiment).

Diagnosis	Patients (%)
Primary ovarian serous cancer (A2, 3, 6, 8, 9, 11, 12, 14)	8 (67)
Primary fallopian tube/ovarian serous cancer (A13)	1 (8)
Primary ovarian mucinous cancer (A15-17)	3 (25)
Experimental screening[a]: A2, 3, 6, 8, 11 and control plasma RP1-6.	11 (5 cancer, 6 control)
Experimental validation: A2, 3, 6, 8, 9, 11-17 and control plasma RP1, 4-6, 10, 12, 13, 18, 22, 27, 28, 33	24 (12 cancer, 12 control)
Parameters	
Malignant ascitic fluid	12 (100)
Grade	
High	12 (100)
FIGO stage	
FIGO I/II: IIc, A13, 14	2
FIGO III/IV: IIIa, A12, 16; IIIb, A8, 11; IIIc, A2, 3, 5, 6, 9, 15	10
Median age	**Years (range)**
Cancer samples (ovarian cancer, ascitic fluid)	66.5 (49–84)
Control samples (healthy controls, plasma)	60.5 (47–77)

FIGO: International Federation of Gynecology and Obstetrics.
[a]Záveský et al. (22).

Table 3. Clinicopathological characteristics of the patients (Urine experiment).

Diagnosis	Patients (%)
Experimental evaluation: UB1-6 (cancer samples), UN1-6 (control samples).	12 (100)
Primary serous ovarian cancer (UB1-6)	6 (100)
Parameters (extracellular urine samples)	12 (100)
Grade	
High: UB1, 2, 4-6	5 (83)
Low: UB3	1 (17)
FIGO stage	
FIGO III/IV: IIIb, UB1, 2, 6; IIIc, UB3-5	6 (100)
Median age	Years (range)
Cancer samples (ovarian cancer-associated urine)	60.5 (57–71)
Control samples (healthy control urine)	64 (57–64)

FIGO: International Federation of Gynecology and Obstetrics.

experiment), Table 2 (Ascites experiment) and Table 3 (Urine experiment).

Clinical samples

All samples were collected using stabilization reagents to ensure the inactivation of RNases. Tissue samples (tumor and normal ovary) were stabilized using RNAlater (Ambion/ThermoFisher Scientific, cat. no. AM7021). Samples of ascitic fluid were collected in Urine Preservation Tubes (Norgen Biotek, Canada, cat. no. 18122). Blood samples were collected in Cell-Free RNA BCT tubes (Streck, USA, cat. no. 218975) for the subsequent isolation of plasma.

Isolation of total RNA

Total RNA was isolated from the tissue samples using a mirVana miRNA Isolation Kit (Ambion/ThermoFisher Scientific, cat. no. AM1560) following the manufacturer's instructions. The samples were weighed, cut into small pieces ($\leq$10 mg) and placed in 10 volumes per unit tissue mass of lysis/binding buffer (e.g. 1 ml of buffer for each 0.1 g of tissue) in a homogenization tube. The samples were briefly disrupted on ice using a Tissue Ruptor homogenizer (Qiagen) and sterile disposable probes. An miRNA homogenate additive (1/10 volume of the tissue lysate/homogenate) was then added, and the mixture was briefly vortexed and then stored on ice for 10 min. Acid-phenol:chloroform equal to the initial lysate volume was added, and the samples were vortexed for 1 min and then centrifuged at 10 000 $\times$ *g* at 4 °C for 5 min to separate the aqueous and organic phases. The aqueous phase was transferred to a fresh tube, 1.25 volumes of 100% ethanol was added and the mixture was briefly vortexed. The samples were then pipetted onto a filter cartridge and centrifuged with three washes. Total RNA was eluted with 100 µl of 0.1 mM EDTA and stored at −80 °C. The RNA was used undiluted for TaqMan Array Human MicroRNA A cards in the screening phase, and diluted RNA was used for the validation phase (15 µl of H_2O added to 20 µl of total RNA).

Total RNA was isolated from the samples of blood plasma and the cell-free ascitic fluid by centrifugation at 1300 $\times$ *g* at room temperature for 15 min and then at 2500 $\times$ *g* at 4 °C for 10 min. Total RNA was isolated using a Plasma/Serum Circulating and Exosomal RNA Purification Maxi Kit, Slurry Format (Norgen Biotek, Canada, cat. no. 50900).

Total RNA was also isolated from cell-free urine supernatant. The urine samples were centrifuged

at 1000 × *g* at room temperature for 10 min, and the supernatants were then centrifuged at 2000 × *g* at 4 °C for 20 min. Total RNA was isolated using a Urine Total RNA Purification Maxi Kit, Slurry Format (Norgen Biotek, Canada, cat. no. 29600).

Reverse transcription

The total RNA from the tissue was reverse transcribed into cDNA in the screening phase using a TaqMan MicroRNA Reverse Transcription Kit and Megaplex RT primers (Human Pool A v2.1, Applied Biosystems/Thermo Fisher Scientific, Foster City, USA). Reverse transcription (RT) in the validation phase (total RNA derived from tissue, ascitic fluid/plasma and urine) used the TaqMan MicroRNA Reverse Transcription Kit and miRNA-specific RT primers following modified manufacturer's instructions, i.e. a scaled-down format (1/2 volume).

Real-time PCR amplification

The reaction mixture for real-time PCR amplification used in the screening phase (Tissue experiment) was loaded onto a TaqMan Array Human MicroRNA A Card (one sample for each array, no pooling, no preamplification, 377 unique human miRNA assays) and consisted of 2× TaqMan Universal PCR master mix, No AmpErase UNG (450 µl), the Megaplex RT product (i.e. cDNA obtained by reverse transcription, 6 µl) and nuclease-free water (444 µl). The screening phase for ascites-derived miRNAs has been performed with preamplification previously using TaqMan Array Human MicroRNA A + B cards containing assays for 754 unique human miRNAs (see (22) for details of the procedure and results).

Real-time PCR amplification in the validation phase was performed in scaled-down reactions (1/2, total volume of 10 µl) in triplicate in 96-well MicroAmp Optical 96-Well Reaction Plates using Xceed qPCR Probe 2× Mix HI-ROX buffer as a master mix (IAB, Czech Republic, cat. no. HPCR10502L). The real-time PCR reactions were run using an Applied Biosystems 7900HT Fast Real-Time PCR System thermocycler (Applied Biosystems/Thermo Fisher Scientific, Foster City, USA).

Experimental design and selection of individual miRNA assays

This study is partly a follow-up of our previous study (22) that comprehensively screened miRNAs derived from ascitic fluid and plasma. We identified 153 miRNAs in that study that were significantly differentially expressed in ascites relative to the controls among 754 miRNAs investigated. The expression of seven miRNAs (miR-200a-3p, miR-200b-3p, miR-200c-3p, miR-141-3p, miR-429, miR-1290 and miR-30a-5p) was validated (22). This pilot study provided a basis for the further evaluation of differentially expressed miRNAs to find congruence between the cell-free ascitic fluid and the tumor tissues. A panel of selected miRNAs was evaluated based on the screening data previously obtained for ascitic fluid (22) and for the present study using independent samples of ascitic fluid and tumor tissue using miRNAs with the most highly dysregulated expression.

We selected eight miRNAs (miR-203a-3p, miR-204-5p, miR-451a, miR-185-5p, miR-135b-5p, miR-182-5p, miR-200b-3p and miR-1290) to be evaluated in the present study excluding miR-200b-3p and miR-1290 in the panel used in the Ascites experiment as these miRNAs have been validated previously (22).

Of the validated miRNAs, three with consistent data (miRs-203a-3p, 135 b-5p and 200b-3p) and five with inconclusive data (miRs-204-5p, 451a, 185-5p, 182-5p and 1290) were included in the validation phase based on the screening phases of the Tissue and Ascites experiments.

Normalization of miRNA expression data

We previously determined that three miRNAs (miR-17-5p, miR-93-5p and miR-425-5p) were best suited as endogenous controls for ascitic fluid/plasma (22) using the geNorm module of qbase+ (Biogazelle, Belgium) (23,24) and BestKeeper algorithm (25), which were also evaluated by geNorm in Tissue experiment in the present study. These miRNAs are among the

most commonly used housekeeping genes for normalization in miRNA studies (26) and were used as normalizers in the validation phase for all three types of samples. Global means were used in the screening phase to normalize the expression data for the TaqMan Array Human MicroRNA cards (for both ascitic fluid (22) and tissue (present study)).

Statistical analyses

qbase+ (24) and MedCalc statistical software (Belgium) were used to analyze the expression data (log-transformed CNRQ data exported from qbase + were used in MedCalc). The data processed in qbase + were corrected for multiple testing using the Benjamini-Hochberg procedure. Two cutoffs of expression level, i.e. Ct <35 and Ct ≤40 were applied in screening experiment with respect to assays in the array with reduced or lacking expression (Ct >35) whereas no cutoff level was applied in validations where Cts were generally low. Mann-Whitney tests were applied to identify differences in expression between the pathological and control samples, and receiver operating characteristic (ROC) curve analyses were used to evaluate the area under the curve (AUC), sensitivity and specificity. p values < .05 were considered significant in all tests.

Results

Tissue experiment

Screening phase

Seven samples of primary serous, mostly high-grade ovarian carcinomas (OC1-7) and seven samples of normal ovarian tissue (ON1-7) were analyzed in the screening phase using TaqMan Array MicroRNA Type A cards. We used two cutoffs of expression level for analyzing the data, i.e. Ct <35 (data group A) and Ct ≤40 (data group B), and the cancer samples were compared with the controls. A total of eight miRNAs were consistently overexpressed in both groups (miRs-200a-3p, 200b-3p, 200c-3p, 141-3p, 429, 182-5p, 224 and 425-5p; see Table 4 for details), and 11 miRNAs were consistently underexpressed in both groups (miRs-532-3p, 486-5p, let-7e, 376c-3p, 574-3p, 140-3p, 143-3p, 199a-3p, 195-5p, 145-5p and 204-5p; see Table 5 for details). Two miRNAs were significantly overexpressed and five were underexpressed in group A relative to the controls (Table S1). Twenty miRNAs were overexpressed and 27 were underexpressed in group B relative to the controls (Table S2). All differentially expressed miRNAs for both cutoffs are presented in Table S3. Expression of the miR-200 family members (particularly miR-141-3p and miR-200a-3p) notably increased the most in both groups.

Validation phase

The differential expression of the eight individual miRNAs was validated for miRs-203a-3p, 204-5p, 451a, 185-5p, 135b-5p, 182-5p, 200b-3p and 1290. miRNA expression was first analyzed and compared between an extensive set of 14 tumor tissue samples (OC1-14; early and advanced stages, low and high grades) and 14 control samples (ON1-14). Seven of the eight selected miRNAs were significantly differentially expressed. Expression relative to the controls was significantly higher for miR-200b-3p (62.7-fold), miR-182-5p (13.5-fold), miR-203a-3p (2.6-fold)

Table 4. Fold differences between tumor tissue and normal ovary in TaqMan Array Human MicroRNA A cards and consistently overexpressed miRNAs (Tissue experiment, screening).

microRNA	FD (Ct ≤40)	p	95% CI, low	95% CI, high	FD (Ct <35)	95% CI, low	95% CI, high	p
hsa-miR-141-3p-4373137	1042.50	.01	420.15	2586.71	684.65	244.04	1920.75	.02277
hsa-miR-200a-3p-4378069	894.43	.01	251.40	3182.22	353.39	146.07	854.94	.02277
hsa-miR-429-4373203	287.91	.02	126.42	655.72	287.91	126.42	655.72	.02277
hsa-miR-200c-3p-4395411	630.65	.01	190.70	2085.59	240.62	91.49	632.87	.02277
hsa-miR-200b-3p-4395362	234.33	.01	74.10	741.02	153.73	56.54	417.97	.02277
hsa-miR-182-5p-4395445	77.74	.01	26.26	230.15	30.99	12.16	79.01	.03207
hsa-miR-224-4395210	12.41	.02	3.78	40.70	9.43	3.76	23.65	.03207
hsa-miR-425-5p-4380926	8.58	.02	3.62	20.34	5.63	2.97	10.68	.02277

FD: fold difference; CI: confidence interval.

Cancer samples OC1-7 were compared with control samples ON1-7. Global mean normalization was applied. Individual miRNA codes are as indicated in the TaqMan Arrays. Two cutoffs of expression level, i.e. Ct <35 and Ct ≤40 were applied in screening experiment. Only significant differences are noted. miRNA nomenclature follows miRBase ID.

Table 5. Fold differences between tumor tissue and normal ovary in TaqMan Array Human MicroRNA A cards and consistently underexpressed miRNAs (Tissue experiment, screening).

microRNA	FD (Ct ≤40)	*p*	95% CI, low	95% CI, high	FD (Ct <35)	95% CI, low	95% CI, high	*p*
hsa-miR-204-5p-4373094	−76.22	.00644	−336.43	−17.27	−43.29	−120.93	−15.50	.03207
hsa-miR-199a-3p-4395415	−11.50	.00644	−25.70	−5.15	−11.52	−26.51	−5.01	.02277
hsa-miR-140-3p-4395345	−10.12	.00644	−28.00	−3.66	−9.43	−15.99	−5.56	.02277
hsa-let-7e-4395517	−10.09	.00644	−28.49	−3.57	−7.11	−21.19	−2.39	.04934
hsa-miR-532-3p-4395466	−10.05	.00644	−28.96	−3.49	−6.74	−13.39	−3.39	.03207
hsa-miR-143-3p-4395360	−9.56	.00644	−23.02	−3.97	−9.57	−27.20	−3.37	.03207
hsa-miR-145-5p-4395389	−8.79	.00644	−18.35	−4.21	−13.40	−35.85	−5.01	.02277
hsa-miR-195-5p-4373105	−8.49	.00644	−15.59	−4.62	−12.94	−31.08	−5.39	.02277
hsa-miR-486-5p-4378096	−7.15	.02252	−26.35	−1.94	−7.00	−20.89	−2.34	.03207
hsa-miR-574-3p-4395460	−5.20	.00644	−11.54	−2.34	−7.93	−18.50	−3.39	.02277
hsa-miR-376c-3p-4395233	−4.87	.01850	−11.27	−2.11	−7.43	−17.57	−3.14	.02277

FD: fold difference; CI: confidence interval.

Cancer samples OC1-7 were compared with control samples ON1-7. Global mean normalization was applied. Individual miRNA codes are as indicated in the TaqMan Arrays. Two cutoffs of expression level, i.e. Ct <35 and Ct ≤40 were applied in screening experiment. Only significant differences are noted. miRNA nomenclature follows miRBase ID.

and miR-135b-5p (2.6-fold) and significantly lower for miR-204-5p (−108.5-fold), miR-451a (−43.3-fold) and miR-185-5p (−2.1-fold). The expression of miR-1290 did not differ significantly. See Table S4 for details.

Expression did not differ significantly between advanced and early stages, but expression for the advanced (Table S5) and early (Table S6) stages were compared to the controls to identify differences. Three miRNAs in the advanced stage were significantly overexpressed (miR-200b-3p, 52-fold; miR-182-5p, 14-fold and miR-135b-5p, 2.2-fold), and three were significantly underexpressed (miR-204-5p, −291-fold; miR-451a, −33.5-fold and miR-185-5p, −2.6-fold) (Table S5). Four miRNAs in the early stage were significantly overexpressed (miR-200b-3p, 72-fold; miR-182-5p, 13.4-fold; miR-203a-3p, 3.4-fold and miR-135b-5p, 3-fold), and three were significantly underexpressed (miR-451a, −52.5-fold; miR-204-5p, −52-fold and miR-185-5p, −1.7-fold) (Table S6). In contrast, a comparison between high- and low-grade samples (Table S7) indicated that one miRNA was significantly overexpressed (miR-182-5p, 3-fold) and two were significantly underexpressed (miR-204-5p, −85.8-fold and miR-135b-5p, −5.5-fold).

miRNA expression between the independent cancer samples (OC8-14) and the control samples (ON8-14) was independently validated to avoid the impact of the samples used during screening. Three miRNAs were significantly overexpressed (miRs-200b-3p, 182-5p and 135b-5p) and two were significantly underexpressed (miRs-451a and 204-5p). miR-203a-3p was previously overexpressed, but not significantly in independent samples ($p = .097$), and miR-185-5p was previously underexpressed, but not significantly in independent samples ($p = .24$). The expression of miR-1290 did not change significantly ($p = .54$). See Figure 1 and Table S8 for details.

ROC curve analysis (tumor versus control tissues)

Sensitivity and specificity were first assessed in the preliminary analyses of the ROC curves using log-transformed CNRQ data for analyzing independent sample sets (cancer samples OC8-14 versus control samples ON8-14) and the differential expression of miRNAs (miRs-200b-3p, 182-5p, 135b-5p, 451a and 204-5p). miRs-200b-3p and 182-5p had the largest AUCs (1.000) and 100% sensitivities and specificities. miR-135b-5p also had a large AUC (0.980), with 100% sensitivity and 85.71% specificity. miR-451a and miR-204-5p had AUCs of 0.939 and 0.878, respectively, and both had a sensitivity of 85.71% and a specificity of 100% (Table S9).

The ROC curves were next assessed for all 28 samples in Tissue experiment (cancer samples OC1-14, control samples ON1-14) to include more samples for obtaining relevant conclusions. miR-200b-3p had a sensitivity and specificity of 100%. miRs-182-5p, 451a and 204-5p had large AUCs (0.995-0.934) and high sensitivities (100-92.86%) and specificities (92.86-100%). miRs-135b-5p and 185-5p had large AUCs (0.847-0.811), high specificity (100-92.86%) and lower sensitivity (71.43%), and miR-203a-3p had the smallest AUC (0.765) and lowest sensitivity and specificity (both 71.43%). See Table S9 for details.

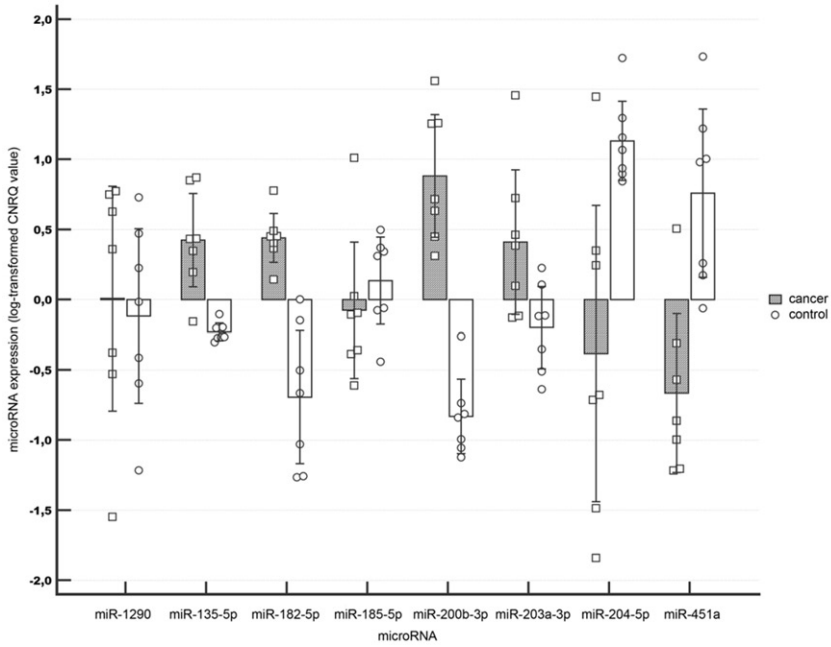

Figure 1. Comparison of miRNA relative expression between tumor and control tissues. Clustered multiple-comparison graph with log-transformed CNRQ (calibrated normalized relative quantities) expression data for independent tumor samples (OC8-14) compared with control samples (ON8-14) in Tissue experiment. Error bars indicate the 95% confidence intervals for the means. Individual cancer samples are indicated as squares, and control samples are indicated as circles. miRs-200b-3p, 182-5p and 135b-5p were significantly overexpressed, and miRs-451a and 204-5p were significantly underexpressed.

Ascites experiment

Screening phase

Five samples of primary ovarian serous cancer (ascitic fluid A2, 3, 6, 8 and 11) and six samples of control plasma (RP1-6) were previously analyzed using TaqMan Array Human MicroRNA A Cards, and four ascitic fluid samples (A6, A7, A9 and A11) and four samples of control plasma (RP4-7) were analyzed using TaqMan Array Human MicroRNA B Cards in the screening phase of our pilot study focused on ascites (22).

Validation phase

We used data from our recent large-scale profiling of ascites-derived miRNAs (22) and data from the present study for tumor tissues to assess the expression of six miRNAs (miR-203a-3p, 204-5p, 451a, 185-5p, 135b-5p and 182-5p) and three candidate endogenous controls (miRs-17-5p, 93-5p and 425-5p). According to our previous data (22), miR-204-5p, miR-203a-3p and miR-1290 (along with miR-200 family members) belonged to the most highly overexpressed miRNAs in ascites relative to plasma whereas miR-451a and miR-185-5p displayed consistent decreases in ascites levels.

Samples of ascitic fluid associated with high-grade ovarian serous and mucinous carcinomas were compared with the control samples of plasma. We first analyzed the expression of extracellular ascites-derived miRNAs using particularly the samples of ovarian serous cancer in the extended sample set along with three mucinous samples (see Table 2). A total of 12 cancer samples were compared with 12 control plasma samples. The mucinous and serous samples did not differ significantly, so we analyzed the serous and mucinous samples together. Three miRNAs (miR-203a-3p, 2791-fold; 204-5p, 1643-fold and 135b-5p, 433-fold) were highly and significantly overexpressed, and miR-182-5p was only weakly (6-fold) but significantly overexpressed, in ascitic fluid relative to the control plasma. miR-185-5p expression was slightly higher in the ascitic fluid (2.2-fold), but not significantly. One miRNA (miR-451a) was significantly underexpressed in ascites (−72-fold). See Table S10 for details.

We next analyzed miRNA levels in independent sample sets to ensure an independent evaluation of expression. Ascitic-fluid samples A9 and 12-17 ($n = 7$) and control plasma samples RP10, 12, 13, 18, 22, 27, 28 and 33 ($n = 8$) were

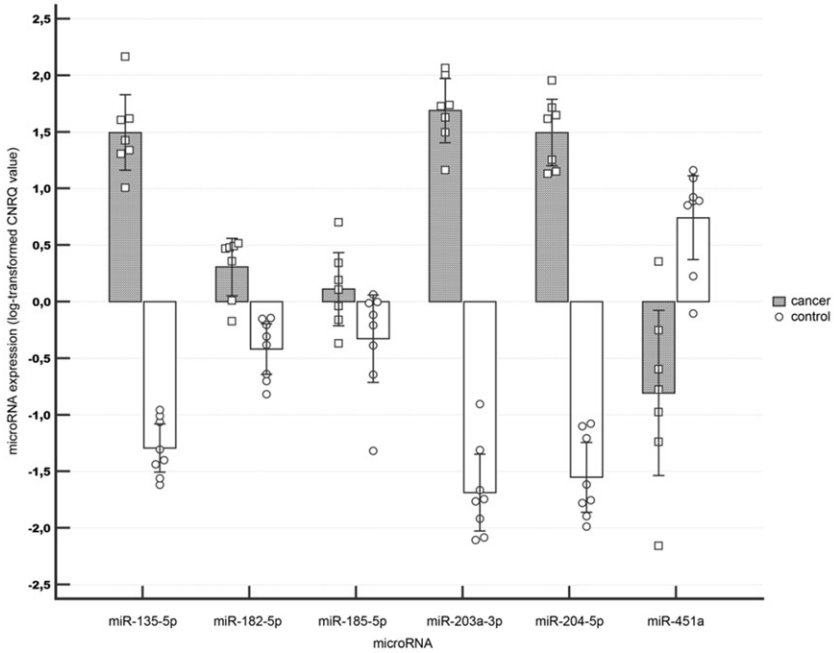

Figure 2. Comparison of miRNA relative expression between ascites and control plasma. Clustered multiple-comparison graph with log-transformed CNRQ (calibrated normalized relative quantities) expression data for independent samples of ascitic fluid (A9, A12-17) compared with control samples (RP10, 12, 13, 18, 22, 27, 28 and 33) in Ascites experiment. Error bars indicate the 95% confidence intervals for the means. Individual cancer samples are indicated as squares, and control samples are indicated as circles. All miRNAs but miR-185-5p were significantly differentially expressed.

included. The results were congruent with those for the extended sample set. See Figure 2 and Table S11 for details.

ROC curve analysis (ascitic fluid versus control plasma)

ROC curve analyses for the differentially expressed miRNAs identified in the statistical analyses were assessed in two sample sets, (1) independent samples not analyzed during screening and (2) all samples included in Ascites experiment (validation). These analyses produced similar significant results. miR-203a-3p, 204-5p and 135b-5p had AUCs of 1.000 and 100% sensitivities and specificities. Two miRNAs (miRs-451a and 182-5p) also had large AUCs (0.964-0.986), sensitivities of 85.71-91.67% and specificities of 100% in both sample sets. See Table S12 for details.

Urine experiment

Expression was evaluated in a panel of eight representative miRNAs (miRs-203a-3p, 204-5p, 451a, 185-5p, 135b-5p, 182-5p, 200b-3p and 1290) and three candidate controls (miRs-17-5p, 93-5p and 425-5p), comparing a small set of cell-free urine samples associated with high-grade serous ovarian carcinomas ($n = 6$) to control urine samples ($n = 6$). These analyses did not find significant differential expression (data not shown).

Discussion

Ovarian cancer is the most malignant gynecologic cancer. Ovarian carcinomas are generally resistant to treatment and often recur, leading to poor outcomes for patients, partly due to late diagnosis in advanced stages in most patients. Screening for ovarian cancer, however, is difficult due to its relatively low incidence, and specific causes and potential triggers remain largely undiscovered. Diagnostic and treatment tools have remained unchanged, despite recent progress. Novel diagnostic, predictive and prognostic biomarkers for ovarian cancer are therefore urgently needed.

The use of miRNAs as potential novel biomarkers has been thoroughly investigated, particularly in ovarian cancer cell lines and tumor tissues, similar to other cancers. A large body of research including also *in vivo* animal studies has identified many candidate miRNA markers

applicable to diagnosis, prognosis and prediction of sensitivity to treatment or as potential treatment targets over the last decade. Such investigations, however, have low reproducibility and limited potential for introduction into clinical practice. So-called liquid biopsies and focus on the real patient samples have therefore been applied to search for clinically more relevant miRNA biomarkers in samples such as plasma and serum, known as circulating miRNAs (7).

Ovarian cancer is a complex, heterogeneous disease capable of escaping both the body's mechanisms of elimination and the diverse anticancer treatments. Ascites (effusion), the accumulation of fluid in the peritoneal (and/or pleural) cavity, has often been associated with ovarian cancer and is a possible medium playing an important role in the spread, invasion and metastasis of ovarian cancer in the peritoneum and omentum and eventually the organs in the peritoneal cavity. The presence of ascites is thus an indicator of poor outcomes, even in early stages. Relapses of the disease, even in patients originally responding to chemotherapy, may be attributed to ascites, because residual cancer cells not removed by surgery may aggregate in this peritoneal fluid and form microscopic tumor spheroids that are more resistant to chemotherapy. The spheroids can then adhere to the surface of organs in the peritoneum and may form new tumors, assisted by chemokines and growth factors in the peritoneal fluid (27). Researchers have previously assumed that the spread of ovarian cancer proceeded directly by the shedding of malignant cells into the intraperitoneal cavity, supported by the novel concept that many ovarian cancers arise in the fallopian tube. Invasion of the lymphovascular space, the occurrence of circulating ovarian cancer cells and experimental evidence, however, suggest that ovarian cancer may also spread hematogenously (28).

We can nevertheless assume that ascites may play important roles in the spread of ovarian cancer based on previous research and clinical data, and future research should also be focused on miRNAs potentially involved in these processes. Identifying potential candidate miRNAs across different kinds of samples, including ascitic fluid, associated with ovarian cancer was therefore the primary objective of the present study.

Ovarian cancer – differential expression of miRNAs in tumor tissues

We identified many miRNAs differentially expressed between tumor tissues and controls in our large-scale profiling of miRNA expression (screening phase) (Table S3). The data for differential expression between screening and validation in Tissue experiment were generally consistent for three overexpressed miRNAs (miRs-200b-3p, 135b-5p and 182-5p) and one underexpressed miRNA (miR-204-5p). Most of the data for miR-203a-3p (overexpressed in all sample comparisons, but not significantly in independent validation) and underexpressed miR-451a (not significantly underexpressed during screening but significantly underexpressed during validation) were consistent. The data for miR-185-5p were inconclusive (overexpressed, but not significantly, during screening, underexpressed during validation and underexpressed, but not significantly, during validation of the independent samples). miR-1290 was not assessed during screening in Tissue experiment, and its overexpression during validation was not significant. See Table S13 for the consistency of the results.

Ovarian cancer – differentially expressed miRNAs in ascitic fluid

Differential expression was similar between screening and validation for overexpressed miRs-203a-3p, 135b-5p and 204-5p assessed in the present study and for miRs-200b-3p and 1290 validated previously (22). One miRNA (miR-451a) was consistently underexpressed during both screening and validation. The data for miR-182-5p (underexpressed during screening, overexpressed during validation) and miR-185-5p (underexpressed during screening, overexpressed, but not significantly, during validation) were inconclusive. See Tables S10, S11 and S13 for details.

Potential functional roles of dysregulated miRNAs across ascitic fluid and tumor tissue

Both ascitic fluid and tumor tissue represent heterogeneous biopsies, potentially comprising effects of various cells, complex origin and surrounding microenvironments, including extracellular compartments, along with active interactions determining biological processes such as carcinogenesis and body defense in space and time. We cannot therefore unequivocally state that an increase in miRNA expression is a characteristic of oncogenesis or that a decrease in expression supports a role of tumor suppressors, because they may represent a simplified model of miRNA functioning. Altered miRNA expression may be due to various mechanisms at different levels, such as chromosomal abnormalities, defects in the machinery of miRNA biogenesis, reciprocal interactions or regulations between individual miRNAs, altered Drosha or Dicer activity, epigenetic changes such as altered DNA methylation or inhibition of histone deacetylase and altered transcription-factor activity (29). These mechanisms were beyond the scope of our study, but further experimental evidence evaluating affected mRNA targets or proteins should provide more insight into the mechanism of dysregulation and its importance in ovarian carcinogenesis.

We evaluated several overexpressed miRNAs. Detailed functional roles of miR-200 family members in ovarian cancer, however, remain unresolved. Oncogenic functions are the most supported, because expression is upregulated across studies, despite the biphasic pattern of expression suggested for the development of metastases linked with the "on/off switches" of the processes known as epithelial mesenchymal transition (30). This family was the most highly overexpressed in our studies, in both tumor tissue and ascitic fluid. The potential function of the miR-200 family as enhancers of ovarian carcinogenesis may thus be assumed.

miR-182-5p was overexpressed during tissue screening and validation and between the high- and low-grade samples of tumor tissue. The data for this miRNA in Ascites experiment were questionable, because miR-182-5p was underexpressed during screening but overexpressed during validation. Oncogenic roles for miR-182-5p in ovarian cancer have nevertheless been reported (e.g. (31,32)).

miR-203a-3p was also overexpressed. miR-203a-3p in tumor tissue was significantly upregulated only when using Ct $\leq$40 in the screening phase but was significantly upregulated relative to the controls in the validation phase (marginally significantly in independent samples). Its overexpression in ascitic fluid was consistent during screening and validation phases. The oncogenic status of miR-203a-3p has been observed in other studies of ovarian cancer (33,34).

miR-135b-5p was the sixth most overexpressed miRNA, including at a cutoff of Ct <35, and was upregulated marginally significantly, including at a cutoff of Ct $\leq$40, in tumor tissue relative to the controls during screening. miR-135b-5p also had the highest upregulated expression when comparing ascitic fluid and plasma at Ct $\leq$40 during screening. This miRNA was only slightly upregulated in tumor tissues relative to the controls but was highly upregulated in ascitic fluid relative to control plasma during validation phases of both experiments. These results suggest an impact of this miRNA in cancer progression in ascitic fluid or a downregulation in the control plasma samples. miR-135b-5p was notably underexpressed in the high- versus low-grade samples of tumor tissues, preventing definite conclusions on the roles of miR-135b-5p in ovarian cancer, as for the lack of other data for miR-135b-5p in this cancer. Data reported for most other cancers suggest that miR-135b-5p may be an oncogenic miRNA (e.g. (35,36)). miR-135b-5p, however, is downregulated in glioblastoma multiforme (37).

miR-451a was the most underexpressed miRNA in ascitic fluid relative to plasma in our recent large-scale screening (22), and the downregulation was confirmed during validation for both ascitic fluid and tumor tissue in the present study. miR-451a was recently reported as downregulated in ovarian tumor tissues (38). Low levels of miR-451a have also been associated with an advanced stage and poor prognosis for ovarian-cancer patients (38). These results should generally be interpreted carefully, because miR-451a is an erythrocyte-enriched marker of

hemolysis (39). The underexpression of miR-451a in tumors nevertheless supports its potential as a tumor suppressor in ovarian cancer.

Our results for miR-204-5p were inconsistent. This miRNA was consistently and significantly downregulated in tumor tissue relative to the controls during both screening and validation but was consistently upregulated in ascitic fluid relative to control plasma, which was also confirmed during validation. The enrichment of miR-204-5p in the ascitic fluid may implicate its roles in cancer progression. miR-204-5p may be upregulated in CD133+ cells (a group of ovarian cancer stem cells) relative to CD133- cells in the OVCAR3 cell line (40). We also cannot exclude its downregulation in the control plasma. The roles of miR-204-5p in ovarian cancer remain to be elucidated, although most studies suggest a role as a tumor suppressor in various cancers (e.g. (41,42)).

Our results for miR-185-5p and miR-1290 also prevent assigning potential functions due to their inconsistent expression. Data are generally lacking, so further research should evaluate the roles of miR-185-5p and miR-1290 in detail.

Conclusion

We comprehensively evaluated miRNA expression in extracellular fractions of ascitic fluid (peritoneal fluid, effusion) and tumor tissue in ovarian cancer. Novel candidate miRNAs were identified among differentially expressed miRNAs, and the corresponding expression patterns in both the ascitic fluid and tumor tissue suggested similar functions in ovarian carcinogenesis and may provide a basis for their evaluation as oncogenes or tumor suppressors. miR-200 family members (miR-200b-3p assessed in detail) were highly overexpressed, supporting their roles as oncomiRs in ovarian cancer. Other validated miRNAs, i.e. miR-182-5p, miR-203a-3p and miR-135b-5p, were also potential candidate oncomiRs. Underexpressed miR-451a may be a candidate for evaluation as a tumor-suppressor miRNA. Candidate extracellular urine-derived miRNAs evaluated here failed to be proved as suitable biomarkers for ovarian cancer.

Further clinical studies using samples of ascitic fluid and tumor tissue in large independent patient cohorts and additional experimental studies are highly warranted to identify miRNAs closely associated with ovarian carcinogenesis, participating in tumor progression, invasion and metastasis and potentially linked to resistance to chemo/radiotherapy. miRNAs identified as potential tumor suppressors, however, may have opposite functions, suppressing ovarian carcinogenesis and dissemination or increasing sensitivity to chemo/radiotherapy treatment. Potential novel miRNA biomarkers based on extensive data should be then applicable to diagnosis, prognosis, prediction of response to treatment or the development of novel therapeutic agents for the management of ovarian cancer.

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Ethical approval

All patients provided an informed consent. The study was approved by the multi-centric Ethics Committee of the General University Hospital in Prague.

Declaration of interest

The authors declare that they have no conflict of interest.

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ORCID

Luděk Záveský http://orcid.org/0000-0001-9592-7535

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