RESEARCH

Evaluation of Cell-Free Urine microRNAs Expression for the Use in Diagnosis of Ovarian and Endometrial Cancers. A Pilot Study

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Abstract Among gynaecological cancers, epithelial ovarian cancers are the most deadly cancers while endometrial cancers are the most common diseases. Efforts to establish relevant novel diagnostic, screening and prognostic markers are aimed to help reduce the high level of mortality, chemoresistance and recurrence, particularly in ovarian cancer. MicroRNAs, the class of post-transcriptional regulators, have emerged as the promising diagnostic and prognostic markers associated with various diseased states recently. Urine has been shown as the source of microRNAs several years ago; however, there has been lack of information on urine microRNA expression in ovarian and endometrial cancers till now. In this pilot study, we examined the expression of candidate cell-free urine microRNAs in ovarian cancer and endometrial cancer patients using quantitative real-time PCR. We compared the expression between pre- and post-surgery ovarian cancer samples, and between patients with ovarian and endometrial cancers and healthy controls, within three types of experiments. These experiments evaluated three different isolation methods of urine RNA, representing two supernatant and one exosome fractions of extracellular microRNA. In ovarian cancer, we found miR-92a significantly up-regulated, and miR-106b significantly down-regulated in comparison with control samples. In endometrial cancer, only miR-106b was found down-regulated significantly compared to control samples. Using exosome RNA, no significant de-regulations in microRNAs expression could be found in either of the cancers investigated. We propose that more research should now focus

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on confirming the diagnostic potential of urine microRNAs in gynaecological cancers using more clinical samples and large-scale expression profiling methods.

Introduction

Ovarian cancer is the most malignant gynaecological cancer, the fifth most common cause of death from cancer in women and the ninth most common female cancer [1]. Most ovarian cancer cases (~61 %) are diagnosed at advanced stages [2]. Apparently, the critical point determining the patient's prognosis is an early diagnosis; currently achieved only in 33 % of cases. While the diagnosis at early stages results in ~70–90 % 5-year patients' survival, late diagnosis is associated with poor prognosis and 5-year survival dropping to 10 – 30 % [2, 3]. Endometrial cancer is the most common malignancy of female genital tract, the fourth most common female cancer and the seventh cause of death among women [1]. The incidence is growing in the last decade significantly in all western countries but the relative 5-year survival (~80–90 %) has not improved over many decades [4, 5]. Many recent scientific efforts are aimed at finding novel diagnostic and prognostic biomarkers for these gynaecological cancers.

MicroRNAs are small noncoding RNAs involved in post-transcriptional regulations of various cellular processes including carcinogenesis [6]. Several profiling studies on microRNAs have been published also for ovarian and endometrial cancers; however, most of the investigations were focused exclusively on tissue specimens and/or cell lines. Recently, the research focus has moved to shed light onto the presence and roles of cell-free miRNAs, particularly as diagnostically applicable biomarkers in plasma and serum [7].

It has been shown recently that cell-free miRNAs may be detected in 12 different normal (i.e., non-diseased) body fluids including urine [8]. Moreover, supernatant urine microRNAs have emerged as potential diagnostic tools in upper urinary tract urothelial cancer and bladder urothelial cancer in this study [8]. A diagnostic potential of urine microRNAs has been further demonstrated particularly in urogenital tract-related diseases. The methods differ in the source of analysed material coming either from released cells (e.g., urothelial cancer [9], prostate cancer [10], bladder cancer [11]), whole urine (bladder cancer [12], prostate cancer [13]), or urine supernatant (bladder cancer [14]).

We suggested that urine miRNAs, particularly as a part of cell-free RNA excreted by kidneys, should reveal a diagnostic potential also in gynaecological cancers. As a proof of

principle, the present study was aimed particularly to test different methods of urine RNA isolation, and to find out whether cell-free miRNAs may be detected and differentially expressed in urine of patients particularly with ovarian and endometrial cancers in comparison with control patients. This is the first study reporting on differential cell-free urinary miRNAs expression in ovarian and endometrial cancers.

Material and Methods

Clinical Samples

Patients with epithelial ovarian cancer, fallopian tube cancer, endometrial cancer, and benign diagnoses undergoing a gynaecological surgery due to suspected ovarian and endometrial cancers were enrolled in this study. Pathological samples of urine were collected in the University Hospital Brno (FN Brno) (designated as "UCB") and in the Institute of the Care of Mother and Child Prague (ÚPMD Praha - Podolí) (designated as "UCP"). Control urine samples were provided by healthy blood donors (post-menopausal women) in the Transfusion Department, General University Hospital Prague (VFN Praha). All patients gave written informed consent. This research was approved by a multicentre Ethical Committee of the General University Hospital Prague. Urine samples (a second morning void) were collected into Urine Collection and Preservation Tubes (Norgen Biotek Corp., Canada). Summaries of clinical samples, diagnoses and representation in the experiments are provided in Table 1.

Pre-Surgery, Post-Surgery and Post-Chemotherapy Samplings

The pre-surgery samples are designated with "A" in the end of the sample code (samples from FN Brno), or without the final letter in the code (samples from ÚPMD Praha). Post-surgery ovarian cancer samples were collected after the surgery and before the application of chemotherapy, and are designed with "B" in the end of the code (exceptional UCB318B is indicated in the Table 1). Post-surgery and post-chemotherapy samples were collected after finishing the whole series chemotherapy treatment and are designated with C in the end of the code. Pre- and post-surgery sampling was applied exclusively in ovarian cancer samples collected in FN Brno.

Isolation of Urinary RNA

As the basic procedure, the protocol for processing the whole urine (Urine Total RNA Purification Maxi Kit, Slurry Format, Norgen Biotek Corp., Canada) was applied according to manufacturer instructions (applied in "preliminary experiment" P1E using whole urine, data not shown). Different centrifugation steps or modifications were included in the following

Table 1 Clinicopathological data summary for patients and samples used in the experiments

Type of sample	FIGO stage	Grade	Age at surgery	Metastases status at surgery	Recurrence (month after surgery)	Death (Month after surgery)	Supernatant-1 experiment	Supernatant-2 experiment	Exosome experiment
Epithelial ovarian cancer							n=9	n=5	n=10
Serous (pre-surgery)							n=5	n=2	n=4
UCB310A	1c	III	53	M0	no	no	●	—	—
UCB318A	3a	III	55	yes, in omentum	yes (10)	yes (12)	●	—	●
UCB417A	3c	III	64	yes, in omentum and peritoneum	no	no	●	●	●
UCP12	3c	III	68	yes, in peritoneum	n/a	n/a	—	●	●
UCB322A (13 days after neoadjuvant chemotherapy)	3a	II	68	yes, in peritoneum	n/a	yes (9)	—	—	—
Serous (post-surgery)							n=2	—	n=2
UCB310B	1c	III	Ibid.	Ibid.	Ibid.	Ibid.	●	—	●
UCB417B	3c	III	Ibid.	Ibid.	Ibid.	Ibid.	●	●	●
UCB322B (23 days after neoadjuvant chemotherapy)	3a	II	Ibid.	Ibid.	Ibid.	Ibid.	—	—	●
Serous post-chemotherapy (disease recurrence)							n=2	n=1	n=2
UCB318B (Chemotherapy 7th day after surgery, sampling B here 10th day after surgery)	3a	III	Ibid.	Ibid.	Ibid.	Ibid.	●	—	●
UCB318C (7 months after surgery, 8 days after chemotherapy)	3a	III	Ibid.	Ibid.	Ibid.	Ibid.	-	●	●
UCB425C (5 months after surgery, 2 months after chemotherapy)	3c	III	56	M0	yes (16)	No	●	—	—
Mucinous (pre-surgery)							n=1	n=1	n=1
UCB315A	1c	II	72	M0	no	No	●	●	●
Mucinous (post-surgery)							n=1	—	—
UCB315B	1c	II	Ibid.	Ibid.	Ibid.	Ibid.	●	—	—
Mixed endometrioid ovarian cancer/endometrioid endometrial cancer (pre-surgery)							—	n=1	n=1
UCP5	3c	III	57	n/a	n/a	n/a	-	●	●
Fallopian tube (pre-surgery)							n=1	—	n=1
UCB902A	3c	II	77	yes, extensively including omentum	yes (15)	no	●	—	—
UCP1	3c	II	62	n/a	n/a	n/a	—	—	●
Fallopian tube (post-surgery)							n=1	—	n=1
UCB902B	3c	II	Ibid.	Ibid.	Ibid.	Ibid.	●	—	●
Endometrial cancer							—	n=5	n=10
Type 1 endometrioid endometrial cancer							—	n=5	n=9
UCP8	1a	II	77	n/a	n/a	n/a	—	●	●
UCP9 (mixed with undifferentiated carcinoma)	n/a	n/a	86	n/a	n/a	n/a	—	●	●
UCP11	3b	n/a	64	n/a	n/a	n/a	—	●	●
UCP13	n/a	n/a	91	n/a	n/a	n/a	—	●	●

Table 1 (continued)

Type of sample	FIGO stage	Grade	Age at surgery	Metastases status at surgery	Recurrence (month after surgery)	Death (Month after surgery)	Supernatant-1 experiment	Supernatant-2 experiment	Exosome experiment
UCP15	n/a	II-III	71	n/a	n/a	n/a	–	●	●
UCP16	3a	I	61	n/a	n/a	n/a	–	–	●
UCP17	n/a	II	55	n/a	n/a	n/a	–	–	●
UCP19	n/a	I	57	n/a	n/a	n/a	–	–	●
UCP21	n/a	I	64	n/a	n/a	n/a	–	–	●
Type 2 endometrial cancer									n=1
UCP22	n/a	III	51	n/a	n/a	n/a	–	–	●
Benign samples – ovarian type	–	–		–	–	–	–	n=2	n=3
UFB01 (ovarian fibroma)	–	–	66	–	–	–	–	●	●
UCP2 (ovarian thecofibroma)	–	–	68	–	–	–	–	–	●
UCP14 (ovarian thecofibroma)	–	–	74	–	–	–	–	●	●
Benign samples – endometrial type	–	–		–	–	–	–	n=2	n=3
UCP7 (endometrial polyp)	–	–	65	–	–	–	–	●	●
UCP10 (endometrial leiomyomas)	–	–	68	–	–	–	–	●	●
UCP18 (intramural tumor)	–	–	53	–	–	–	–	–	●
Healthy controls	–	–	–	–	–	–	n=13	n=12	n=13

Involvement of the samples within the particular experiment is indicated with black circles, otherwise hyphen indicates that sample is not involved/not assessed

three experiments prior to total RNA isolation using the mentioned kit. In the Supernatant-1 experiment (further "S1E"), urine samples were centrifuged for 10 min at 100 x g at RT, and then supernatant was centrifuged at 500 x g for 10 min at RT. In the Supernatant-2 experiment (further "S2E"), urine samples were centrifuged for 10 min at 300 x g at 4 °C, and then supernatant was centrifuged at 2,000 x g for 20 min at 4 °C. Stronger centrifugation steps were applied for an efficient elimination of potential cell contaminants. In the Exosome experiment, urine samples were processed according to a manufacturer protocol for Urine Exosome RNA Isolation Kit (Norgen Biotek Corp., Canada) to isolate pure exosome RNA. Prior to isolation, urine samples were centrifuged at 1,000 x g, for 10 min at RT and stored at 2 °C to 8 °C until further processing. Two additional centrifugations were then applied, the first at 300 x g, for 10 min., at 15 °C, and the second at 2,000 x g, for 10 min, at 15 °C. Next, supernatant was filtered through a sterile, 0.2 μm PVDF filter (Whatman Puradisc 13 mm, GE Healthcare Life Sciences) to 15-ml tube to ensure isolation of vesicles up to 200 nm.

Assessing RNA Quantity and Quality

RNA concentration and quality were inspected using Nanodrop 1000 spectrophotometer (Thermo Scientific), and Agilent RNA 6000 Pico Kit (alternatively Agilent RNA 6000 Nano Kit, Agilent Small RNA kit) on Agilent 2100 Bioanalyzer (Agilent Technologies), the latter also for observation of potential cellular contamination. For assessing potential DNA and protein contamination, Qubit fluorometer (Life Technologies) and Qubit DNA BR Assay kit and Qubit Protein Assay kits were employed.

Reverse Transcription and Real-Time PCR Analyses

Single-stranded cDNA was generated from total RNA using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) following manufacturer's protocol and scaled-down (1/2, total volume 7.5 μl) reaction volumes. For each sample, the same input of total RNA volume was used. Each RT-reaction involved 2.5 μl RNA (S1E and S2E), or 4.6 μl RNA in the Exosome experiment. PCR amplifications were performed in scaled-down reactions (1/2, total volume 10 μl) in triplicates (S1E and S2E), or duplicates (Exosome experiment) and contained 0.7 μl cDNA from reverse transcription (S1E and S2E), or 1.85 μl cDNA (Exosome experiment). Real-time PCR was carried on an Applied Biosystems 7900HT thermocycler (Life Technologies). No-template controls, no-real-time PCR controls, and inter-plate controls were included in the analyses. PCR amplification reactions were incubated in a 96-well optical plate using the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and followed

by holding at 4 °C. Expression data were captured using SDS 2.4 and RQ 1.2.1 software.

Real-Time PCR microRNA Expression Data Analyses

Real-time PCR Miner [15] was employed to obtain efficiency-corrected data and results are presented as coming from this type of analyses. Within the verifications, Cy0 [16] and delta Ct methods were applied. Relative expressions of microRNAs were calculated using the following equations in Real-time PCR Miner, Cy0, and delta Ct methods. First, adjusted efficiency was calculated. It was 1 + mean efficiency of genes (Real-time PCR Miner), or 2 in Cy0 and delta Ct methods. Then, this adjusted efficiency was powered to Ct (i.e., $(1+E)^{Ct}$ in Real-time PCR Miner data), or using Cy0, or Ct value. Next, relative R0 expression was calculated (relative $R0 = 1/(1+E)^{Ct}$). Geometric mean was used as the normalization factor. Alternatively, individual microRNA (or a combination) was selected for normalization based on BestKeeper [17] and NormFinder [18] calculators, for further verification of results. Data were log-transformed prior to statistical analyses.

Statistical Analyses

Statistical processing of log-transformed data was done in MedCalc Statistical Software. According to assessment of normality, data were further analysed using non-parametric tests (Wilcoxon test for paired samples, Mann–Whitney test) when normal distribution of data was not achieved. Parametric alternative tests were applied in case of data normality (paired samples t-test, independent samples t-test). P-values less than 0.05 were considered statistically significant.

Results

Urine microRNAs Tested as Candidate Diagnostic Markers

In total, 18 individual miRNAs were tested across all the experiments, 11 assays revealed as acceptable. These microRNAs revealed with lacking or compromised expression and are not included in this study: hsa-let-7d, hsa-miR-181a, hsa-miR-9, hsa-miR-34c, hsa-miR-302a, hsa-miR-214, hsa-miR-127-3p.

Cell-Free Urine miRNAs Expression in Ovarian Cancer (Supernatant-1 Experiment)

MicroRNA Expression in Pre-Surgery Versus Post-Surgery Ovarian Cancer Samples

There were no statistically significant differences between pre- and post-surgery ovarian cancer/tube cancer samples

(UCB310, UCB315, UCB318, UCB417 and UCB902 samples) in 10 out of 11 microRNAs tested: miR-21, miR-223, miR-92a, miR-200b, miR-16, miR-29a, miR-367, miR-106b, miR-100, miR-20a and miR-1228. In miR-16, there was a statistically significant decrease in post-surgery samples ($P=$ 0.0461). We next excluded the data for UCB318A and UCB318B, as the latter sample was exceptionally collected after the chemotherapy. Then, the differences between pre- and post-surgery samples in miR-16 (and also in other miRNAs) did not appear statistically significant.

Differences in microRNA Expression between Pre-surgery Ovarian Cancer and Control Samples

Initially, we analysed statistically all ovarian cancer samples (UCB310A, UCB315A, UCB318A, and UCB417A) plus fallopian tube cancer (UCB902A). The results showed four miRNAs significantly differentially expressed between ovarian cancer and control samples (miR-92a, miR-200b, miR-106b and miR-100). The miR-92a and miR-200b were up-regulated, and miR-106b along with miR-100 were down-regulated in cancer samples in contrast to control samples (Table 2, suppl. file 1a).

Data were further analysed for various sample combinations. We evaluated expression of miR-92a, miR-200b and miR-106b in cancer samples versus controls for a) serous ovarian cancers (UCB310A, UCB318A, UCB417A; b) serous ovarian cancers (the same set as in a)) plus fallopian tube cancer (UCB902A). In both samples sets, the significant up-regulation of miR-92a along with miR-200b, and down-regulated expression of miR-106b was confirmed. Interestingly, miR-100 was not found down-regulated significantly in the serous cancer set ($P=0.0693$), but this difference was significant ($P=0.0415$) in the set of serous cancers with the fallopian tube cancer. We additionally extended the serous ovarian cancer set using also data for post-chemotherapy sample

Table 2 Overview of significantly de-regulated microRNAs in ovarian cancer in comparison with controls (S1E experiment)

MicroRNA	Change in expression (cancer vs controls)	Fold-difference	P-value
miR-92a	up-regulation	31.97	0.0009
miR-200b	up-regulation)*	24.54	0.0013
miR-106b	down-regulation	0.27 (minus 3.7-fold)	0.0026
miR-100	down-regulation	0.30 (minus 3.3-fold)	0.0266

Pre-surgery ovarian cancer/fallopian tube samples included (UCB310, UCB315, UCB318, UCB417 and UCB902). Data based on Real-time PCR Miner analyses, and normalized to geometric mean

*Up-regulated expression of miR-200b detected by Real-time PCR Miner could not be confirmed by alternative processing algorithms

UCB425C (as this patient suffered from recurrence) along with other serous cancers UCB310A, UCB318A, UCB417A. Results for miR-92a, miR-106b and miR-200b appeared congruently with previous results, but miR-100 was not significantly de-regulated.

To further enlarge the sample set, we also added adjusted data for two patients from the S2E experiment to the S1E data set. This resulted in a confirmation of above-mentioned results for miR-106b and miR-200b (miR-92a was not assessed in S2E and could not be analysed), but not for miR-100 (see section Combined Cell-Free miRNAs Expression of Ovarian Cancer Samples (Supernatant-1 and Supernatant-2 Experiments)). Mean fold-differences of cancer samples versus control samples for S1E experiment are presented in Table 2, and suppl. file 1a.

Cell-Free Urine miRNAs Expression in Ovarian and Endometrial Cancers (Supernatant-2 Experiment)

Ovarian and endometrial cancers along with four benign samples and one mixed ovarian/endometrial cancer were analysed in this experiment (Table 1). Analysed miRNAs were: miR-21, miR-223, miR-200b, miR-16, miR-29a, miR-367, miR-106b, miR-100, miR-20a and miR-1228. Data are presented as the fold-changes in relation to control samples (Suppl. file SF1b).

Differences in microRNA Expression between Ovarian Cancer and Control Samples

There was miR-106b identified as the prominent miRNA being significantly down-regulated in various combinations of ovarian cancer samples in Supernatant-2 experiment (see Supp. file 1b). We exceptionally involved the sample UCB318C (lack of remission, disease recurrence, early death 12 months after surgery) instead of the true pre-surgery sample not available (urine of this sample was reserved for experiment Exosome) into data sets as this sample represents the pathological state in a patient. In the following comparisons, miR-106b retained capacity to discriminate cancer samples a) UCB315A, UCB318C (post-surgery, post-chemotherapy), UCP12, and mixed ovarian and endometrial cancer UCP5 ($P=0.0036$), b) UCB315A, UCB318C, UCP12 ($P=0.0094$) and c) UCB315A and UCP12 ($P=0.0285$), from control samples. However, these results are based only on limited numbers of ovarian cancer patients representing different histological subtypes. Therefore, we combined the normalized and adjusted data from Supernatant-1 and Supernatant-2 experiments for ovarian cancer samples to analyse more samples adding two ovarian cancer samples (UCP5 and UCP12) to Supernatant-1 data set. These analyses confirming the previous results are shown in the

section Combined Cell-Free miRNAs Expression of Ovarian Cancer Samples (Supernatant-1 and Supernatant-2 Experiments).

Differences in microRNA Expression between Endometrial Cancer and Control Samples

Endometrial cancer samples of type 1, i.e., UCP8, UCP9 (mixed with undifferentiated carcinoma), UCP11, UCP13 and UCP15 were compared with control samples. Expression analyses revealed only miR-106b to be significantly down-regulated in cancer samples ($P=0.0087$). The results for this miRNA remained identical after an exclusion of UCP9 sample to eliminate the impact of its mixed histology on results ($P=0.0053$).

Combined Cell-Free miRNAs Expression of Ovarian Cancer Samples (Supernatant-1 and Supernatant-2 Experiments)

In order to involve more ovarian cancer samples in statistical analyses, we performed a logarithmic regression analysis to establish a regression equation between the two experiments. Data from Supernatant-1 and Supernatant-2 control samples (identical patients) yielded significant ($P<0.0001$) regression equation $y=1.2572+1.1664 \log(x)$, where y=Supernatant-1, and X=Supernatant-2. Next, we adjusted the Supernatant-2 data for two ovarian cancer samples (one mixed ovarian with endometrial cancer = UCP5, one pure ovarian cancer UCP12) to be added to Supernatant-1 sample set. This new data set for ovarian cancers was then compared with control samples in various combinations, confirming the previous results for miR-106b and miR-200b, but not in miR-100 (Note: miR-92a was not assessed in the Supernatant-2 experiment).

These combinations of samples were tested in comparison with controls: a) all pre-surgery ovarian cancers (four serous, one mucinous), fallopian tube cancer plus mixed ovarian/endometrial cancer ($n=7$); b) all pre-surgery ovarian cancers (four serous, one mucinous) plus fallopian tube cancer ($n=6$); c) pre-surgery ovarian serous cancers ($n=4$); d) pre-surgery serous ovarian cancers plus fallopian tube cancer ($n=5$); e) pre-surgery ovarian serous cancers plus post-chemotherapy sample UCB425C (patient with the recurrence) ($n=5$). All above-mentioned sample combinations yielded invariably identical results: significant down-regulation of miR-106b and significant up-regulation of miR-200b. miR-100 could not be confirmed as down-regulated significantly in either sample combination.

Expression of microRNAs in the Experiment Exosome

In this experiment, different sets of clinical samples were investigated using exosomal RNA, combining particularly ovarian cancer samples ($n=10$) and endometrial cancer samples ($n=10$) (Table 1). Analysed miRNAs were: miR-21, miR-200b, miR-16, miR-29a, miR-106b, miR-20a, miR-1228. Two other miRNAs, miR-100 and miR-367 had a compromised appearance.

Differences in microRNA Expression between Pathological and Control Samples

First, ovarian cancer and endometrial cancer samples were compared with controls. We also compared benign, control, and malignant samples. No significant difference was found in either group for any microRNA investigated.

Fold-Differences in microRNA Expression between Pathological and Control Samples

Although differences were not significant in the statistical analyses, the expression pattern (fold-differences to control samples) deserves an attention. For particular microRNA, there were both increases and decreases of miRNA expression in comparison of pathological samples with control samples (data not shown), except for miR-100 with suspect, invariably down-regulated expression in several samples.

A Compromised Expression of Exosome miRNAs

Based on our data, it could be assumed that isolation of pure exosome miRNAs fraction may affect negatively the expression results, giving many missing values and differences between experiments (data not shown) may potentially suggest alterations among various fractions of urine microRNAs due to their involvement in various lipoprotein complexes or carrier vesicles. Moreover, we could not find differential miRNA expression in samples previously giving promising results in S1E and S2E experiments.

Verification of Results for S1E, S2E and Exosome Experiments

Alternative normalization procedures and alternative real-time PCR expression data processing algorithms (Cy0, delta Ct methods) were used as the two basic types of verifications of the abovementioned results in S1E, S2E experiments. For the Exosome experiment, alternative normalization approach was used. In ovarian cancer (S1E experiment), we could confirm up-regulated miR-92a, and down-regulated miR-106b along with miR-100. miR-200b could not be confirmed as up-regulated miRNA definitely, and miR-223 appeared as potentially up-regulated miRNA in ovarian cancer. In endometrial cancer (S2E experiment), the down-regulated expression of miR-106b was confirmed. Interestingly, here also the miR-29a appeared down-regulated in endometrial cancer and up-regulated in ovarian cancer. Further, miR-21 was found

potentially up-regulated in endometrial cancer. We could not find any microRNA de-regulated in the Exosome experiment.

Discussion

The gynaecological cancers including ovarian and endometrial cancers form a group of less studied cancers. MicroRNAs coming from urine of patients with these diagnoses have not been investigated yet. In the present study we obtained the results indicating that 1) microRNAs could be expressed in all cell-free fractions of urine studied; 2) there were mostly no significant differences between pre- and post-surgery ovarian cancer samples; 3) expressions of several microRNAs were significantly different between pathological and control samples (but not in exosome RNA), 4) methodology is feasible for the clinical utilization particularly due to a stabilization of urine in special tubes at ambient temperature.

Within the S1E experiment based on ovarian cancer urine samples, we found miR-92a (and potentially miR-223, in the verification) up-regulated significantly, and miR-106b down-regulated significantly in ovarian cancer. In the S2E experiment, significantly down-regulated expression of miR-106b was found in urine associated with ovarian and endometrial cancers, potentially (in the verification) also with up-regulated expression of miR-21 in endometrial cancer. The increased expression of miR-92a is consistent with previous reports finding its up-regulation in ovarian cancer tissues [19] and serum [20]. Similarly, the decreased expression of miR-106b is comparable with other investigations on ovarian cancer tissues [21], whole blood [22], or plasma [23]. On the other hand, miR-106b was found up-regulated in recurrent ovarian cancer [24].

In endometrial cancer, we found miR-106b down-regulated. Previous experimental evidences have shown contradictory functions of this miRNA (tumor suppressor gene, and oncogene) in various cancers. For endometrial cancer, there is generally lack of information on miR-106b expression, but miR-106b has been showing an association with invasive endometrial cells while being under-expressed [25]. Recently, down-regulation of miR-106b has been demonstrated to result in MMP2 over-expression, consequently leading to the increased invasion and metastasis in breast cancer [26].

The miR-29a was found (potentially) down-regulated in endometrial cancer and potentially up-regulated in ovarian cancer in the S2E experiment. This is consonant with previous studies both in endometrial cancer [27] and ovarian cancer (e.g., [21]). The down-regulated expression of miR-100 and up-regulated expression of miR-200b in ovarian cancer could not be confirmed definitely. The down-regulated expression of miR-100 in our major ovarian cancer urine set in S1E (but not significant in different sample combinations) appeared

congruently with other reports on down-regulation in ovarian cancer tissues (e.g., [19, 24]).

Up-regulated expression of miR-200b is corresponding with many previous studies in ovarian cancer (e.g., [28]) but miR-200b has been showing some level of controversy in ovarian cancer investigations (see [3]).

Further research will be needed to validate urine microRNAs in large patients' cohorts using large-scale profiling approaches. It will also be necessary to elucidate intrinsic and extrinsic factors associated with alterations in global urine miRNAs excretion, as well as in various RNA fractions occurring in urine. We suggest that urine miRNAs may serve as novel diagnostic biomarkers in gynaecological cancers.

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