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MiRNA expression in plasma extracellular vesicles of prostate cancer patients after radical prostatectomy

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Abstract: Aim: Radical prostatectomy (RP) is the most frequent frontline PCa treatment. Biochemical recurrence (BCR) after radical prostatectomy occurs in 20%–40% of patients, but only 30% of these patients demonstrate cancer progression. Sensitive and specific markers of RP effectiveness are needed. Cell-free miRNAs from blood plasma packed in extracellular vesicles (EVs), namely the expression of 14 miRNAs before and one week after RP, were studied in comparison with their expression in EVs of benign prostatic hyperplasia patients and healthy donors in the present manuscript. **Materials and methods:** Plasma EVs isolation was performed using an aggregation-precipitation protocol. MiRNA was isolated using the Guanidine isothiocyanate/Octanoic Acid Protocol. MiRNAs expression was assessed by reverse transcription and quantitative RT-PCR. **Results:** It was shown that 11 of the 72 studied miRNA ratios changed significantly after RP. Moreover, one of two miRNAs (miR-125b and miR-30e) took part in each miRNA ratio whose relative expression changed after RP. **Conclusion:** RP causes differential expression of plasma EVs miRNA. The obtained results indicate the prominent role of miR-125b and miR-30e in response to radical therapy. The study of miRNA expression in dynamics and in different biofluid fractions is required to assess the potential of extracellular miRNAs as sensitive biomarkers of therapy and to select their optimal source.

Keywords: prostate cancer; miRNA; liquid biopsy; blood plasma; prostatectomy; extracellular vesicles

1. Introduction

Prostate cancer (PCa) is a prevalent malignancy, with 1,414,259 new cases in 2020 [1]. The accompanying socio-economic burden is huge [2]. Radical prostatectomy (RP) is the gold

standard and the only surgical treatment for localized PCa [3,4]. Post-RP biochemical recurrence (BCR) used for monitoring of PCa treatment efficacy is defined as a minimum two PSA values of 0.2 ng/mL or higher. To date, no more sensitive, specific, and avowed marker than PSA informed on RP clinical relapse or RP effectiveness exists. However, BCR after a radical prostatectomy or adjuvant therapy, whose assessment starts 1 month after surgery and then is done every 3rd month, occurs in 20%–40% of patients [5,6]. At that point, it has been proved that biochemical failure is not equal to clinical relapse, and only about 30% of BCRs are related to metastasis of a distant organ [7]. Incomplete removal of the tumor, insufficient skill of the surgeon, and individual characteristics contribute to relapse development. Classical predictors of BCR include positive surgical margins and their specific characteristics: size, number, location, and Gleason score at the margin [8,9]. However, 16.2% and 30% of patients with negative surgical margins still face BCR at 5 and 10 years after RP, respectively [8]. In addition, 53% and 36% of patients with positive surgical margins do not develop BCR at 5 and 10 years after RP [8]. These facts indicate that a more sensitive and specific marker of PCa relapse is needed. The biological characteristics of tumor cells and their surroundings can also increase the risk of PCa relapse. Thus, along with PSA, other markers related to tumor cell phenotypes and aggressiveness are required to predict the efficacy of RP (alone or combined with subsequent treatment) and post-RP disease-related events.

There are several options for patients with BCR, including active surveillance, salvage radiation, androgen deprivation therapy (ADT), high intensity focused ultrasound therapy, as well as various combinations of hormonal, radiation, and chemotherapy [10,11]. The costs of PCa therapies are rising more rapidly than those of other oncology type nowadays. Implementing individualized patient profiles and adapted treatment algorithms would make treatment costs more transparent providing a “road map” for the cost reduction [2,12].

It should be mentioned that RP affects not only the oncological process but also the patient’s health and standards of living, including blood loss and transfusion rates, additional procedures such as pelvic lymph node dissection, and may lead to serious postoperative complications. They include various symptoms, from long-term urinary incontinence or retention and erectile dysfunction to neurapraxia, reoperation, and mortality [13], some of which manifest in up to 80% of patients [14]. Therefore, the prediction of the RP effectiveness before the procedure and thus the selection of the “responders” for RP will lead to a faster recovery, a reduction in possible side effects of therapy, and an improvement in their quality of life. It should be mentioned that patient status may vary and “non-responders” to RP may change their status after treatment to “responders” thus enabling optimization of RP in terms of its efficacy and patient comfort.

The trend towards personalized medicine induces looking for novel diagnostic/prognostic markers, including molecular biological ones. In this respect, cell-free miRNAs may represent a pool of prognostic markers measured in postoperative tissues and bodily fluids. The latter may represent RP efficacy markers as well as a one-time prognosis and long-term monitoring. Nevertheless, the dynamics of miRNA expression after RP remain insufficiently investigated [15].

Previously, it was shown that the expression of miRNAs, which regulate genes involved in the development of prostate cancer, in urine EVs has great potential as a diagnostic marker of PCa and changes significantly after RP [16–18]. EVs for such studies are usually derived from biofluids via ultracentrifugation; however, this technique, due to the complicated and expensive equipment, cannot be used in clinical laboratories. To overcome this limitation and reveal if cell-free miRNA from EVs are to be related the RP procedure, we study their expression in EVs isolated from biofluids via the aggregation–precipitation approach [19] with subsequent miRNA isolation and quantification. Despite the obvious benefits of urine as a source of diagnostic material, blood is nevertheless demanded by doctors as a habitual source of such material obtained after bleeding in a routine test.

The current study is devoted on the analysis of the expression of 14 miRNAs in extracellular blood plasma vesicles before and after RP in comparison with their expression in EVs of benign prostatic hyperplasia patients and healthy donors. These 14 miRNAs were selected based on our microarray study using a custom miRCURY LNA miRNA qPCR panel (Exiqon, Vedbaek, Region Hovedstaden, Danmark) as well as RT-PCR [15,18,20] and study of the manuscripts devoted to study PCa-related miRNAs [21,22]. The study was approved by the ethics committee of ICBFM SB RAS (№10, 22.12.2008). Written informed consent was obtained from all participants. All experiments on human subjects were conducted in accordance with the Declaration of Helsinki.

2. Materials and methods

2.1. Sample collection

Table 1. Overview of the study population.

		Healthy donors, N = 33	**BPH patients, N = 30**	**PCa patients, N = 22**
Age	Mean ± SD Range	52.3 ± 4.9 45–60	62.5 ± 9.34 47–80	62.2 ± 5.7 54–74
PSA, ng/mL	Mean ± SD	0.7 ± 0.1	9.6 ± 6.3	7.4 ± 0.7 before RP; 0.14 ± 0.32 year after RP
TNM	T1N0M0 T2N0M0			28% 72%
Gleason score	5 6 7	-	-	0% 50% 50%

Blood samples from 33 healthy male donors (HD), 30 patients with benign prostatic hyperplasia (BPH), and 22 PCa patients before and after RP were obtained from E.N. Meshalkin National Medical Research Center of the Ministry of Health of the Russian Federation (Novosibirsk, Russia) involved in the study. The age range and mean age, blood PSA levels, disease stage, and Gleason score (for PCa patients) of the study population are shown in Table 1. Blood PSA levels were additionally assessed 1 year after RP.

Samples from PCa patients after RP were collected on the day of release (averagely the 7th day after RP). All steps from blood sampling to obtaining the EVs fraction were performed in accordance with the recommendations described in the miSEV guidelines [23].

Venous blood was collected in EDTA-sprayed-coated vacutainers, stored at 4 ℃, and processed within 4 h. To obtain blood plasma, samples were sequentially centrifuged at 400 g for 20 min and at 800 g for 20 min, both at 4 ℃. To withdraw cellular debris, the samples were centrifuged at 17,000 g at 4 ℃ for 20 min.

2.2. Isolation of plasma EVs

Plasma EVs isolation was performed by an aggregation-precipitation protocol using dextran blue and polyethylene glycol (PEG), as described previously [19]. The pellet was resuspended in PBS (500 μL), frozed in liquid nitrogen, and stored at –80 ℃ for subsequent miRNA isolation.

2.3. Isolation of miRNA by the Guanidine isothiocyanate/Octanoic Acid Protocol

Before isolation of miRNA, EVs samples were thawed and softly mixed. Guanidine isothiocyanate/Octanoic Acid miRNA isolation from urine and blood plasma was performed as described previously [19]. Isolation of blood plasma EVs was carried out as described for plasma. After the addition of denaturation buffer, synthetic cel-miR-39-3p was spiked in the samples at 50,000,000 copies per isolation. RNA precipitation by isopropanol was performed as described previously [19]. Air-dried miRNA pellets were dissolved in 30 μL of RNAse-free water.

2.4. Reverse transcription and quantitative RT-PCR

Reverse transcription (RT) of miRNA templates was performed as described by Chen *et al.* [24]. Primers and probes for reverse transcription and TaqMan qPCR (view Supplementary Materials) were synthesized in the Laboratory of Medicinal Chemistry (ICBFM SB RAS, Novosibirsk, Novosibirsk region, Russia). Samples without RNA templates (instead of a matrix, the sample contains water) were used as negative controls. Real-time PCR was performed on the CFX 96TM Real-Time System (Bio-Rad CFX Manager 3.1, Bio-Rad Laboratories, USA). All reactions were performed in duplicate in a total volume of 24 μL. Each reaction contained a specific TaqMan probe (see Supplementary Materials, Table S1). Threshold cycle (Ct) values of the assessed miRNAs were compared in samples from different donor groups. MiRNA expression was evaluated in 2 sets – miR-30e, -125b, -200b, -205, -660, -375, -19b, -92a, -31 and cel-miR-39-3p due to technical restrictions and miR-30e, -125b, -19b, -378a, -425, -222, -144, -22.

2.5. Statistical analysis

Statistical analysis was conducted using Statistica 6.0 software. Ct values were used to perform ratio-based normalization, efficaciously assessing the relative expression of all possible combinations of any two miRNAs in the sample [25,26]. Due to the fact that miRNA

expression was assessed in 2 sets of 9 miRNAs, normalization was used within each set. Thus, 72 miRNA ratios were obtained. For each ratio, the Ct difference (dCt) values and the difference in dCt values, the mean dCt and its standard deviation were calculated. The normality of the distribution was analyzed using the Shapiro-Wilk test.

Comparisons between groups were performed using the non-parametric Kruskal-Wallis test, Friedman test followed by Post-Hoc analysis using Tukey's correction. Correlations between miRNA expression ratios and clinicopathological parameters were analyzed using Spearman criteria.

3. Results

3.1. MiRNA selection

Previously we publish the data of cell-free miRNA expression by microarray [20] and selected set of miRNA which were differentially expressed in healthy donors and PCa patients considering medians and data distribution features (hsa-miR-19b, -22, -92a, -222, -378a, -425, -30e, -31, -125b, -200b, -205, -375, -660). 12 miRNA from this set were verified (hsa-miR-19b, -22, -92a, -378a, -425, -30e, -31, -125b, -200b, -205, -375, -660). Looking through the literature data we uncovered that miRNA-144 (was not selected from microarray data by our criteria) and microRNA-222 also demonstrated high diagnostic efficacy and thus these miRNAs were included to the panel with the intention to enhance its reliability [21,22]. Examples of RT-qPCR curves are shown in the supplementary materials (Figure S1).

3.2. Analytical characteristics of research methods

For all miRNAs, qRT‑PCR assays with a working range of 24–38 threshold cycles (Ct) of PCR were designed. All presented data were obtained using RNA samples that produced Ct values within the working range of the systems. Cel-miR-39(-3p) was used as an internal standard (spike in control). This miRNA was added to the probes after RNAses inactivation on the isolation step. Thus we obtained control of the isolation and RT and PCR. Spike-in control was detected in all samples at 25 ± 1 Ct. The dCt of miRNA pair ratio in every group was not normally distributed (Shapiro-Wilk test); thus, a nonparametric Kruskal-Wallis test followed by Post-Hoc analysis using Tukey's correction was used for data analysis.

3.3. Influence of radical prostatectomy on miRNA expression in plasma EVs

Eleven miRNA pairs significantly altered their relative expression after radical prostatectomy (Table 2, $p < 0.05$). The trend of miRNA ratio changes after RP was compared with the differences between PCa patients and both control groups (healthy donors and BPH patients). The miR-125b/miR-378a ratio after RP became close to its value for BPH donors and did not significantly differ from it (Table 2, line 5). In other ratios, the difference between PCa patients and control groups after RP became more pronounced (Table 2).

Table 2. Effect of radical prostatectomy on miRNA levels in plasma EVs of PCa patients. The data are presented as the ratio of two miRNAs before and after RP compared with those in BPH and healthy donors.

	miRNA pair	PCa dCt after RP vs. before RP	dCt before RP vs. HD	dCt after RP vs. HD	dCt before RP vs. BPH patients	dCt after RP vs. BPH patients
1	miR-22/ miR-30e	–1.8*				–2.1**
2	miR-125b/ miR-425	–1.3*				no difference
3	miR-222/ miR-30e	–1.2*		no difference	no difference	–2.7**
4	miR-144/ miR-30e	–1.2*				–2.7***
5	miR-125b/ miR-378a	–1.2*		–1.2 **	1.6***	
6	miR-125b/ miR-92a	–1.2**	no difference	no difference	no difference	no difference
7	miR-125b/ miR-19b	–1.1**		–1.3**		
8	miR-378a/ miR-30e	–1 *			–2***	–3***
9	miR-30e/ miR-92a	–0.7*		no difference		
10	miR-125b/ miR-30e	–0.5*			no difference	no difference
11	miR-660/ miR-30e	0.5*				

«*» – $p < 0.05$, «**» – $p < 0.01$, «***» – $p < 0.001$, Friedman test

3.4. Correlations between miRNA expression ratios and clinicopathological parameters

Using Spearman criteria, correlations between miRNA expression ratios and clinicopathological parameters were analysed. The ratios of 11 miRNA pairs correlated with the age of donors (Table 3).

All observed correlations were weak. There was no significant correlation between miRNA ratios before or after RP and patients' clinical characteristics, including PSA level or tumour progression, before or 1 year after RP. However, the studied group of post-RP patients was uniform: all of them were BCR negative except for one patient, who was characterized by a PSA level of 1.5 ng/mL and secondary changes in the pelvic lymph node. That is the reason why no correlations were observed.

Table 3. Correlation between the miRNA ratio and age of the participants of the study.

miRNA ratio	Age	
	r	p
125b/19b	–0.3420	<0.001
19b/92a	0.3548	<0.001
200b/19b	–0.3513	<0.001
205/19b	–0.3565	<0.001
22/19b	–0.2836	<0.001
22/378a	–0.2335	<0.05

Table 3. *Cont.*

miRNA ratio	Age	
	r	p
30e/19b	–0.3936	<0.001
30e/92a	–0.2306	<0.05
375/19b	–0.2749	<0.01
425/19b	–0.2307	<0.05
660/19b	–0.4343	<0.001

4. Discussion

Almost 30% of PCa patients experience BCR after RP, and approximately 24%–34% of patients with BCR develop metastasis [27,28], whereas BCR-negative patients do not relapse with unique exceptions. Produced mainly by the glands in the transitional zone of the prostate, PSA is associated with benign prostatic hyperplasia and may indirectly indicate the tumour tissue volume and growth of metastases induced by PCa tumour cells.

Cancer treatment causes significant alterations in miRNA expression in patient biofluids [18,29–31]. Such alterations have been demonstrated for all typical therapeutic strategies for prostate cancer, including radical prostatectomy [15]. Due to the vast involvement of miRNAs in processes of prostate cancer development as well as their stability in biofluids, miRNA-based liquid biopsy is a potential tool not only for diagnosis but also for therapy effectiveness assessment [32]. Among other characteristics, prospective markers of therapy effectiveness should change after (or during) therapy, predict tumour relapse, and be independent of therapy side effects. However, the initial step in the discovery of prognostic markers is to assess their alterations in patient samples during and after therapy. In fact, there is still a limited number of studies aimed to research miRNA expression changes after RP. For example, miR-320a, -b , and -c expression levels in plasma increased after RP in the group of patients without relapse and did not change in the group of patients with biochemical recurrence [16]. In other studies, it was shown that RP did not cause a significant change in the expression of miR-200b and miR-375 [17,33], which is supported by the facts revealed in the current study.

In this study, 11 different miRNA ratios changed significantly after RP. However, alteration of miRNA expression after radical prostatectomy may be related to various reasons, including not only the decrease in tumour burden but also the surgery itself and its side effects. The fact that one of two miRNAs (miR-125b and miR-30e) take part in each miRNA ratio whose relative expression changed significantly after RP indicates a valuable role for these miRNAs in response to radical therapy. According to the literature, miR-125b is a tumour suppressor miRNA whose altered expression is an early event in tumourigenesis [34,35]. miR-125b is involved in the cell cycle regulation, proliferation, and apoptosis [36–38]. Here, we have demonstrated that the relative expression of this miRNA in blood EVs (miR-125b/miR-19b, miR-125b/miR-30e, miR-125b/miR-378a, miR-125b/miR-425, and miR-125b/miR-92a) increased after RP, and this may indicate that increased expression of miR-125b could be considered a therapy efficacy

marker. In contrast, miR-30e is known as a tumour growth promoter. In addition, there have been reports of a possible role for miR-30e as a prognostic marker in the development of BCR [39]. In the current work, the relative expression of miR-30e (miR-144/miR-30e, miR-222/miR-30e, miR-22/miR-30e, and miR-378a/miR-30e) in blood EVs decreased after radical prostatectomy, obviously because of the reduced miR-30e expression. The above described phenomena may occur due to tumour removal and reflect the remission process. Interestingly, miR-30e was involved in 4 miRNA ratios that differed not only before and after RP but also between PCa patients after RP and BPH donors. The observed difference may be related to the surgical removal of the hyperplastic tissue. However, the fact that all miRNA ratios that changed significantly after RP did not differ significantly between healthy donors and PCa patients before RP suggests that these changes are more likely associated with RP than with the remission process. First, this may be due to the heterogeneity of the groups and a large scatter of the data in healthy donors, and second, to the fact that the processes regulated by the studied miRNAs do not appear in healthy people, but are developed in PCa patients, in other words, with the background of the oncological process. The last is indirectly confirmed by the data on the comparison of altered miRNA expression after RP with that in BPH samples.

The correlation analysis did not show any significant correlation between miRNA ratios before and after RP and patients' clinical characteristics, including PSA levels before or after 1 year. Thus, PSA levels and miRNA ratios are not closely related and can be considered independent prognostic markers.

We previously analyzed the influence of RP on relative miRNA expression in urine, blood plasma, and urine EVs [18]. Urine EVs were characterized by the greatest number of the most prospective potential markers of therapy effectiveness within the analyzed biofluid fractions [18] as well as compared with blood EVs, which were analyzed in the present study. As mentioned above for miR-125b and miR-30e, their expression ratios significantly changed after RP in urine EVs to the level of healthy donors [18]. The observed difference indicates that miRNA ratios from plasma EVs are inferior to those from urine EVs as a source of therapy effectiveness markers. It seems that the observed phenomenon is specific for PCa, which does not exclude the possibility that plasma miRNAs (both from EVs and from the supernatant) might be more effective as markers of therapy effectiveness for tumours of other localizations. It should be mentioned that pools of urine and blood miRNAs are generated by different sets of cell types. Plasma miRNAs are generated by a significantly larger number and types of cells, which can lead to an "alignment" of miRNA levels due to the generation by cells with high and low production. In this regard, it is important that these data are valid for blood plasma and not coincide with miRNA expression in urine. Thus, in the context of selected miRNA sets, blood plasma is not a superior source of PCa diagnostic material. This conclusion of ours is supported by the review by Jain *et al.* [40].

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Supplementary data

The authors confirm that the supplementary data are available within this article. Figure S1. Examples of RT-qPCR curves. Table S1. Sequences of primers and probes used for reverse transcription and TaqMan qPCR.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical statement

The study was performed in accordance with the Declaration of Helsinki and approved by the ethics committee of Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences (22.12.2008, №10). All individuals provided written consent before participation.

Authors' contribution

Conceptualization: M.K., O.B. and P.L.; data curation: P.L., I.O.; formal analysis: M.K.; investigation: A.Y.; methodology: A.Y., M.K., O.B., I.O.; resources: I.O.; supervision: P.L.; visualization: M.K.; writing–original draft: M.K.; writing–review & editing: M.K., O.B. and P.L.; project administration: P.L.; funding acquisition: O.B. All authors have read and agreed to the published version of the manuscript.

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