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Looking for miRNA from plasma microvesicles differentially expressed in prostate tumors and healthy men

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Abstract: Aim: Current prostate cancer (PCa) diagnostics are based on PSA level assessment as well as histological analysis of biopsies. However, both false negative and false positive diagnoses are still made. Sensitive and specific markers of PCa are needed. In the present manuscript 14 different cell-free miRNAs were studied in blood plasma extracellular vehicles (EVs) of PCa and benign prostatic hyperplasia (BPH) patients as well as healthy donors. Materials and Methods: Plasma EVs isolation was performed by an aggregation-precipitation protocol. miRNA was isolated by the guanidine isothiocyanate/octanoic acid protocol. miRNAs expression was assessed by reverse transcription and quantitative PCR. Results: It was shown, that the expression of 2 miRNA ratios: miR-30e/miR-19b and miR-19b/miR-92a, differed between all groups. 8 miRNA ratios differentiated PCa patients and healthy donors, 15 ratios differentiated BPH and PCa patients, as well as BPH patients and healthy donors. 12 miRNA pairs aberrantly expressed in blood EVs of PCa and BPH patients were characterized by ddCt values over 1,0. Moreover, three of these miRNA ratios (miR-378a/miR-30e, miR-378a/miR-144, and miR-660/miR-19b) were characterized by high values of AUC (79%–82%), sensitivity (70%–78%) and specificity (63%–81%). Conclusion: The diagnostic characteristics of studied miRNA ratios indicate that they cannot be considered an independent and sufficient diagnostic marker but, quite likely, can be used as additional indicators if a diagnosis is needed to be clarified. Their potential as additional markers to PSA is substantiated by the lack of significant correlation between these markers.

Keywords: prostate cancer; miRNA; liquid biopsy; blood plasma; benign prostatic hyperplasia; extracellular vesicles

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1. Introduction

Prostate cancer (PCa) remains to be among the main causes of oncological morbidity and mortality [1] despite considerable advances in its diagnosis and treatment. At the first stage, for diagnosis of PCa PSA level, digital rectal examination, transrectal ultrasonography are usually used. However, all these three methods are characterized by low specificity or sensitivity, thus, the final diagnosis is made using biopsy. The discrimination of PCa from benign prostatic hyperplasia (BPH) is prominent challenge face clinicians while diagnosis of PCa. BPH, a noncancerous enlargement of the prostate gland, is the most common benign disease found in men and accompanying PCa [2,3]. Currently, PCa and BPH are discriminated using histological analysis of tissues. Indications for a prostate biopsy include determination of the area of compacted tissue during a digital examination, organ changes detected by ultrasound examination, increased PSA levels in the blood. For men under forty, the threshold of PSA is 2.5 ng/mL, and for the elderly—4.5 ng/mL. With age, the prostate enlarges and, as a result, the production of PSA increases. For bioptates analysis, 12 core biopsies are needed. This amount of material is sufficient and complete information about the tumor state of the prostate gland [4]. However, 10%–30% of false negative diagnoses occur when multifocal biopsy is used [5–7], not to mention false positive diagnoses (2.2% [8]). Moreover, biopsy is an invasive procedure with the risks of various side effects like hematuria, pain in the rectum and perineum, hemospermia, acute prostatitis, bleeding from the rectum, acute urinary retention, acute orchiepididymitis [4]. The development of sensitive liquid biopsy based markers is a perspective alternative to invasive biopsy, which may prevent unnecessary biopsies and related health challenges as well as false negative results.

Extracellular miRNAs were shown to be involved in all crucial steps of tumor development: cell cycle regulation, apoptosis, angiogenesis, epithelial-mesenchymal transition, invasion, adhesion, DNA repair, metastasis *etc.* [9–11]. Combined with relative stability of miRNAs in biological fluids and active secretion by cancer cells miRNAs attract the attention as potential biomarkers for cancer diagnostics, in particular, PCa diagnosis. It must be mentioned that extracellular miRNAs are present in all biofluids and a significant part of miRNA are packed in extracellular vesicles (EVs). EVs are membrane-covered nanoparticles produced by cells which take part in the intercellular communication via horizontal transfer of biologically active molecules like proteins and nucleic acids, including miRNAs. Tumor cells may use EVs to influence surrounding tissue, for example to activate angiogenesis, as well as to affect distant organs, for example by formation of premetastatic niches [12–14]. One of the issues to develop EVs based diagnostics is to select proper biofluid as a source of EVs. In the case of prostate tumors, blood plasma and urine have the highest potential.

So far as blood and blood plasma are widely used in clinical diagnostics and thus are a common object of study for clinician, in the current study we tried to evaluate if 14 miRNA from blood plasma microvesicles are to be a useful source of miRNAs for PCa diagnostics. Previously the high potential of similar miRNAs set isolated from urine EVs for PCa

diagnosis was shown [15–17], however, their expression in plasma EVs as well as their diagnostic potential while analyzed in this biofluid remain poorly studied.

2. Materials and methods

2.1. Sample collection

Blood samples from 27 BPH and 33 PCa patients were obtained from the E.N. Meshalkin National Medical Research Center of the Ministry of Health of the Russian Federation (Novosibirsk, Russia). Blood samples from 33 healthy male donors (HD) were obtained from blood donors in the blood transfusion department. Each donor was interviewed by a doctor about the presence of any complaints from the genitourinary system, the presence of cancer in the donor or his relatives (and a corresponding questionnaire consisting of a total of 59 questions was filled out). If the PSA level in a donor blood exceeded age limits, this donor was excluded from the study.

The age data, PSA, the prostate cancer stage, and the Gleason score of the study population are provided in Table 1. The study was approved by the ethics committee of ICBFM SB RAS (No. 10, 22.12.2008). Written informed consent was provided by all participants. All experiments on human subjects were conducted in accordance with the Declaration of Helsinki.

Table 1. Characteristics of the study population.

		Healthy donors, N = 33	**BPH patients, N = 27**	**PCa patients, N = 33**
Age	Mean ± SD Range	52.3 ± 4.9 45–60	61.5 ± 8.9 47–80	60.8 ± 7.6 51–84
PSA, ng/mL	Mean ± SD Range	0.9 ± 0.9 0.1–2.8	8.1 ± 4.2 2.1–18.2	11.6 ± 9.4 1.3–37.6
	Clinical range [18]	40–49 yr ≤ 2.5 50–59 yr ≤ 3.5 60–69 yr ≤ 4.5 70–79 yr ≤ 6.5	-	-
TNM	T1N0M0 T2N0M0	-	-	17% 83%
Gleason score	6 7	-	-	39% 61%

Venous blood was collected in EDTA vacutainers, stored at 4 ℃. Samples were centrifuged at 400 g for 20 min and then at 800 g for 20 min, at 4 ℃. Cellular debris was removed by centrifugation at 17,000 g, 4 ℃ for 20 min.

2.2. Isolation of plasma EVs

To obtain plasma EVs, 500 μL of human blood plasma was mixed with 1.25 mL of 1 M NaCl, 0.377 mL PBS, 0.124 mL 1 M TrisHCl (pH 7.0), 0.1 mL DEXB (0.1 mg/mL), and 150 μL of PEG solution (25% PEG 20000 in PBS) and incubated for 30 min at 4 ℃ [19].

The samples were then centrifuged at 17,000 g, 20 min. The pellet was resuspended in 500 μL PBS and stored at −80 ℃.

2.3. Isolation of miRNA by Gu/OcA protocol

MiRNA isolation from blood plasma EVs was performed using Gu/OcA protocol as described for plasma by Lekchnov *et al.* [20]. Synthetic cel-miR-39-3p was spiked in the samples at 5×10^7 copies per isolation. Air-dried miRNA pellets were dissolved in 30 μL of RNAse-free water.

2.4. Reverse transcription and quantitative RT-PCR

Reverse transcription (RT) on miRNA templates was performed as described by Chen C. [21]. Primers and probes for reverse transcription and TaqMan qPCR (Supplementary Table S1) were synthesized in the Laboratory of Medicinal Chemistry (ICBFM SB RAS, Novosibirsk). Samples without RNA templates were used as negative controls. Real-time PCR was carried out on the CFX 96TM Real-Time System (Bio-Rad, USA). All reactions were carried out in duplicate in a total volume of 24 μL. Each reaction contained a specific TaqMan probe. Threshold cycle (Ct) values of assessed miRNAs were compared in samples from different donor groups. The miRNA expression was evaluated in 2 sets: miR-30e, -125b, -200b, -205, -660, -375, -19b, -92a, -31 and miR-30e, -125b, -19b, -378a, -425, -222, -144, -22 and cel-miR-39 due to technical restrictions.

2.5. Statistical analysis

Statistical analysis was carried out with Statistica 6.0 software. Threshold cycle (Ct) values were used to perform ratio‐based normalization [22,23]. MiRNA expression was evaluated in 2 sets of 9 miRNAs, thus, normalization was made within each group. Accordingly, 72 miRNA ratios were set up. For every ratio, Ct difference (dCt) values, the mean dCt, its standard deviation and the difference of dCt values were calculated. The normality of the distribution was analyzed using the Shapiro-Wilk test. Comparisons between groups were done with the non-parametric Kruskal Wallis test followed by Post‐Hoc Test analysis using Tukey's correction. Correlations of miRNA expression ratios with clinicopathological parameters were analyzed using Spearman criteria.

3. Results

Reliable PCa (as well as other types of cancer) diagnostics could not be done using single miRNA marker. To form a panel of miRNAs for PCa diagnostics cell-free miRNA from healthy and PCa patients were compared by microarray [15,24]. Microarray data were analysed using the asymptotic Wilcoxon-Mann-Whitney Test and Benjamini-Hochberg correction to adjust the statistical significance for multiple comparisons. 12 miRNA from this set were verified (hsa-miR-19b, -22, -92a, -378a, -425, -30e, -31, -125b, -200b, -205, -375,

-660) in subsequent study [16] and 2 additional miRNA demonstrating good diagnostic efficacy by other authors [25,26], were additionally introduced in the panel.

For all miRNAs, qRT-PCR assays with a working range of 24–38 Ct of PCR were used. Only RNA samples that produced Ct values within the working range of the systems were taken in to analysis. Spike-in control (cel-miR-39) was detected in all samples at 25 ± 1 Ct. Analyzed miRNA ratios were not characterized by a normal distribution of dCt values in all analyzed groups (Shapiro-Wilk test).

3.1. Correlations of miRNA expression ratios with clinic-pathological parameters

Using Spearman criteria correlations of miRNA expression ratios with clinicopathological parameters were analyzed. Ratios of 12 miRNA pairs correlated with the age of donors and only four and five miRNA ratios correlated significantly with PSA level and Gleason score correspondingly. However, these correlations were weak: the coefficient didn't exceed 0.27 for PSA and 0.54 for Gleason score (data not shown).

miRNA ratios associated with TNM stage were not found. All observed correlations were weak.

3.2. The diagnostic potential of plasma EVs miRNAs

Tables 2 and 3 demonstrate the results of statistical analysis of miRNA ratios based on the difference in dCt values (ddCt). Only statistically significant differences are shown. Using one-way ANOVA the significant effect of the donor diagnosis factor on 22 miRNA ratios was shown. These miRNA pairs were analyzed by Tukey post-hoc test for pairwise comparisons. As a result, miRNA ratios, in which expression levels in extracellular vesicles of blood plasma differed significantly between different groups of donors, were identified (Table 2).

Table 2. Comparison of mean dCt values for differentially expressed miRNA ratios in groups listed. ddCt values are presented in the table.

	miRNA ratio	PCa vs HD	PCa vs BPH	BPH vs HD
1	30e/19b	–1.1*	1.0*	–2.1**
2	19b/92a	0.8*	–0.6 *	1.4**
3	660/19b	–0.8*		–1.3**
4	378a/425	1.4**		2.4**
5	125b/378a	1.0*		–1.8**
6	222/30e		–1.9**	2.3**
7	378a/30e		–2.8**	3.5**
8	144/30e		–1.6**	1.8**
9	31/30e		–2.5**	2.3**
10	31/660		–2.4*	1.9*
11	22/378a		2.2**	–2.5**
12	205/200b		–1.4*	
13	205/30e		–1.8*	1.5*
14	378a/144		–1.2**	1.7***
15	378a/222		–1***	1.3***
16	205/19b	–1.3*		
17	125b/19b	–0.9*		

Table 2. *Cont.*

	miRNA ratio	PCa vs HD	PCa vs BPH	BPH vs HD
18	425/19b	–2.2*		
19	30e/425		1.8*	
20	125b/660		–1.2*	
21	205/660		–1.3*	
22	222/22			–1.2*

«*»: $p < 0.05$, «**»: $p < 0.01$, «***»: $p < 0.001$, Kruskal-Wallis test
PCa—prostate cancer, HD—healthy male donor, BPH—benign prostatic hyperplasia

Table 3. The diagnostic potential of blood EVs miRNAs for PCa and BPH. miRNA ratios differentiating PCa and BPH patients.

PCa patients vs	**HD**			**BPH**			**Both HD and BPH**		
miRNA ratio	AUC	sensitivity	specificity	AUC	sensitivity	specificity	AUC	sensitivity	specificity
miR-30e/ miR-19b*	72%	57%	70%	70%	64%	67%	53%	54%	50%
miR-125b/ miR-378a	64%	76%	55%	64%	76%	56%	52%	62%	57%
miR-19b/ miR-92a	72%	62%	70%	72%	74%	77%	52%	50%	57%
miR-378a/ miR-144	70%	66%	74%	70%	66%	67%	51%	52%	50%
miR-660/ miR-19b	70%	64%	78%	70%	64%	67%	53%	51%	50%
BPH patients vs	**HD**			**PCa**			**both HD and PCa**		
miR-30e/ miR-19b	80%	63%	94%	62%	63%	55%	75%	77%	71%
miR-144/ miR-30e	78%	67%	78%	72%	67%	79%	75%	70%	74%
miR-19b/ miR-92a	90%	78%	82%	72%	67%	73%	81%	70%	79%
miR-378a/ miR-30e	90%	81%	91%	76%	63%	79%	83%	78%	79%
miR-378a/ miR-144	88%	85%	85%	70%	74%	65%	80%	74%	77%
miR-660/ miR-19b	86%	78%	87%	70%	78%	64%	78%	78%	75%

*Similar miRNA pairs are in bold

Thus, expression of 2 miRNA ratios: miR-30e/miR-19b and miR-19b/miR-92a differed between all groups. 8 miRNA ratios differentiated PCa patients and healthy donors, 15 ratios differentiated BPH and PCa patients, as well as BPH patients and healthy donors (Table 2).

Roc-curve analysis revealed ratios miR-30e/miR-19b, miR-125b/miR-378a, miR-19b/miR-92a,miR-378a/miR-144, and miR-660/miR-19b that differentiated PCa patients from HD and BPH patients with a specificity around 70% and sensitivity up to 77%. The same miRNAs differentiated PCa patients from a united group of HD and BPH patients with specificity that did not exceed 57% and a sensitivity of no more than 57%.

The same miRNA pairs plus miR-144/miR-30e and miR-378a/miR-30e demonstrate better discriminating capacity for differentiation of BPH patients from HD (sensitivity and specificity up to 90%), PCa patients (sensitivity and specificity up to 67% and 69%,

respectively) and mixed groups including HD and PCa (sensitivity and specificity 78 and 79%, respectively for miR-378a/miR-30e).

4. Discussion

As it was already noted in the introduction, identification and differentiation of PCa and BPH are the important points in PCa diagnostics. Considering the limitations and missed points of biopsy, not to mention on other currently used diagnostic approaches, a liquid biopsy is the most adequate alternative. miRNA, circulating in blood may represent such a liquid biopsy diagnostic marker [27]. It is known that miRNAs circulating in microvesicles represent the best source of PCa diagnostic miRNA due to the mode of miRNA generation and the potentiality to select a more cancer specific population of circulating miRNA [16,28,29].

In present work the relative expression of 14 miRNAs assembled into 72 ratios in plasma EVs from patients with PCa, BPH, and healthy men were investigated. Our study did not use a single normalizer, since earlier studies, including those performed by us, showed the best diagnostic potential of microRNAs when using the analysis of their ratios [22,23].

The significant difference between groups was observed for 22 different miRNA ratios. The most significant differences between groups were observed between BPH patients and healthy donors, as well as between PCa and BPH patients, which is an undeniable advantage of the presented analysis. 15 different miRNA ratios were shown to differ significantly between PCa and BPH patients. The most reliable and robust miRNA ratios from a diagnostic point of view are characterized by a high ddCt value. 12 of 15 miRNA pairs aberrantly expressed in the blood EVs of PCa and BPH patients were characterized by a ddCt value over 1.0. Moreover, three of these miRNA ratios (378a/30e, 378a/144, and 660/19b) were characterized by high values of AUC (79%–82%), sensitivity (70%–78%) and specificity (63%–81%). The high potential of blood plasma as a source of valuable PCa markers is also supported by literature data. For example, aberrant expression of miR-19b in BPH tissues and blood plasma was shown earlier [30,31].

In addition to the above, we found 8 miRNA pairs differentially expressed in HD and PCa patients, but ddCt values exceeded 1.0 only for 4 different miRNA ratios: 30e/19b, 378a/425, 205/19b, and 425/19b. The sensitivity and specificity of these miRNA ratios were not high enough (Table 3), although changes in their expression seem to be associated with the development of PCa, since differences between PCa patients and both HD and BPH are observed, as well as a fundamental decrease in these parameters when the comparison groups are merged (Table 3). With an increase in the specificity level up to 100%, the sensitivity of all analyzed miRNA ratios drops significantly, which does not allow the use of the approaches for the formation of a diagnostic panel of miRNAs proposed earlier [15]. Of course, a ddCt value less than or equal to 1.0 is not a good indicator and, accordingly, the use of these miRNA ratios cannot be considered an

independent and sufficient diagnostic marker but, quite likely, can be used as additional indicators if a diagnosis is needed to be clarified.

However, it should be mentioned that 6 miRNA ratios may be used for differential diagnostics of BPH. Moreover, the sensitivity and specificity of these miRNA pairs are significantly higher, however, they are still insufficient to form a diagnostic panel. In addition, the versatility of the miRNAs used and their potential as diagnostic markers, regardless of the degree of damage to the prostate gland, are evidenced by only insignificant correlations between the level of relative miRNA expression and age, PSA, and Gleason parameters.

Earlier, we analyzed the profile of the same set of miRNA in urine, including urine EVs, of PCa, BPH patients, and healthy donors. It was found that miRNA from urine EVs is characterized by the highest diagnostic potential (up to 100% sensitivity with absolute specificity) [16]. Thus, blood appears to be a less suitable source of diagnostic miRNAs compared to urine EVs. This may be due to the fact that blood miRNAs are a more heterogeneous population generated by many cells from lots of body tissues. At the same time, it is known that glomerular filtration from blood plasma is limited by the permeability of the basement membrane and the slit diaphragms between the "legs" of podocytes. Complexes with a diameter of no more than 8 nm [32] can pass into the lumen of the nephron, and microvesicles circulating in the blood cannot penetrate the 30 Å glomerular barrier. Although large shuntlike pores of radius 110–115 Å were described, their number is very low [33]. At the same time, the anatomical structure of the genitourinary system facilitates the penetration of biological material, including microvesicles, from the prostate gland into the urine.

5. Conclusion

Summarizing all above, in the present work, 15 miRNA ratios were found to differ in PCa and BPH patients. Three of these miRNA ratios (378a/30e, 378a/144, and 660/19b) were characterized by the highest ability of EVs to differentiate PCa and BPH patients. At the same time, based on our previously published works, it is still necessary to note the fact that it is the microvesicles of urine, rather than blood, that contain a larger number of diagnostic miRNAs, and diagnostics based on their analysis have a significantly higher sensitivity and specificity. On the basis of the data obtained with regard to PCa, we would recommend using miRNA from urine EVs as a diagnostic material [16], especially since the biomaterial (urine) sampling procedure is absolutely non-invasive, obtaining the EVs fraction is not difficult [19] and can be performed in medical laboratories of any level of equipment, as well as the procedure for isolating miRNAs from EVs [20].

Supplementary data

The authors confirm that the supplementary data are available within this article.

Acknowledgments

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Conflicts of interests

The authors have no conflicts of interest to declare.

Ethical statement

The study was performed in accordance with the Declaration of Helsinki and approved by the ethics committee of ICBFM SB RAS (No. 10, 22.12.2008). Written informed consent was provided by all participants.

Authors' contribution

Concept: 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: O.B. and P.L. All authors have read and agreed to the published version of the manuscript.

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