ORIGINAL ARTICLE

PLASMA EXOSOMAL miRNA SCREENING FOR THE DISCOVERY OF NOVEL BIOMARKERS IN PROSTATE CANCER. A PILOT STUDY

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ABSTRACT

Objective/Purpose: Prostate cancer (PCa) is one of the leading causes of cancer deaths in men worldwide, and there is an urgent need of new, more specialized diagnostic biomarkers in this field. MicroRNAs (miRNAs) are small, non-coding RNA species, which post-transcriptionally regulate gene expression. They have been documented in various types of cancer as being differentially expressed relative to controls, including in extracellular vesicles (EVs) such as exosomes. **Materials and methods**: Expression levels of a miRNA panel were analyzed using qRT-PCR in plasma exosomes of PCa patients and healthy controls, with three normalization methods. **Results:** Two miRNAs were differentially expressed in a statistically significant manner ($p<0.05$) in PCa patients' exosomes vs. controls, considering all three normalization methods (miR-1972 and miR-590-5p). The area under the curve (AUC) was 0.808 in both cases, and the specificity and sensitivity for miR-590-5p were 0.6 and 0.933, respectively, and for miR-1972 it was 0.733 and 0.8, respectively. **Conclusion:** This study contributes preliminary data to new PCa biomarker discovery; however, it needs of future validation in larger studies to confirm the biomarker potential of these miRNAs.
Keywords: MicroRNA, prostate cancer, exosomes, gene expression

INTRODUCTION

Prostate cancer (PCa) is one of the leading causes of cancer mortality in men, representing the second cause of cancer deaths worldwide [1]. According to the American Cancer Society, the key statistics for PCa show that in the U.S. alone, there are more than 30.000 deaths due to PCa in 2019 until now, and 1 out of 9 men will be diagnosed with PCa during his lifetime [2]. Furthermore, this type of malignancy becomes a major health concern due to population aging worldwide [3].

Currently, PCa is diagnosed based on prostate specific antigen (PSA) testing, digital rectal examination (DRE) which are paired with Gleason's score and other clinicopathological factors (age, clinical tumor stage). All of the above have proven to be either non-specific (PSA) or less sensitive (DRE), leading not only to inconsistency or late diagnostic, but also to overdiagnosis due to the non-specificity of PSA [4,5]. Therefore, new possible biomarkers for PCa diagnosis are being extensively studied nowadays, which could overcome the disadvantages of the diagnostic strategies presently in use. There is mounting evidence showing that a number of small endogenous molecules called miRNAs could aid in the biomarker discovery for this type of malignancy [6-9].

MiRNAs are a class of small (about 22 nucleotides in length), non-coding RNA molecules that regulate the expression of protein-coding genes at the translational level [10,11]. Although they were firstly discovered for having an essential role on the larval development of C.elegans [12], their role as biological regulators began to be recognized in the early 2000s, when studies have shown that miRNAs are involved into a series of physiological processes, such as cellular differentiation, proliferation, apoptosis [11]. Later findings have proven their involvement in cancer, showing that more than half of the known miRNAs are located in cancer-associated genomic regions [8,13].

Recently, miRNA expression profiling studies are being extensively investigated, highlighting the importance of miRNA analysis from biological fluids, turning the minimally invasive, liquid biopsy approach into a new optimized diagnostic strategy [14]. MiRNAs studies have also shown that these molecules are found in extracellular vesicles (EVs), such as exosomes, and they could be utilized due to the advantages regarding stability and quantity of miRNA that they enclose [15].

In our miRNA screening study, we aimed to analyze the expression levels of a number of plasma exosomal miRNAs from patients diagnosed with PCa, compared to healthy controls.

MATERIAL AND METHODS

Subject population and sample preparation

This study included a total of 30 subjects, out of which 15 were patients with confirmed prostate adenocarcinoma and 15 are healthy, cancer free controls. Subject recruitment was done in in the Urology department of the Emergency County Hospital in

Timisoara, Romania. All subjects provided informed consent and the study received the approval by the Ethics Committees of the Emergency County Hospital and the University of Medicine and Pharmacy "Victor Babes", in Timisoara, Romania.

After an overnight fasting, venous blood was sampled and collected in EDTA-containing tubes. Samples were centrifuged after collection (1500g, 10 min) for plasma separation, which was then transferred into 1,8 ml cryotubes and immediately frozen at -80°C.

Exosomal RNA extraction

Plasma exosomes were isolated using the Total Exosome Isolation reagent kit (Life Technologies), according to manufacturer's instructions. Total RNA was subsequently extracted from the previously isolated exosomes, using Total Exosome RNA and Protein Isolation Kit (Life Technologies).

MiRNA expression profiling

Expression analysis of multiple miRNAs was performed with the qRT-PCR method, using the microRNA Ready-to-Use PCR and Human Panel I+II, V3.M (Exiqon) panels. This kit contains two 384-well plate miRNA analysis panels. We used three normalization strategies to increase the confidence of the results (exogenous cel-miR-39 spike in method, global mean normalization and the NormFinder algorithm). QRT-PCR reactions were performed in an ABI PRISM® 7900HT Sequence Detection (Life Technologies) system. After processing the data, we selected only the miRNA species which were present in at least 80% of the samples at a Ct cutoff value of 37. We used the same sample volumes for all patients and controls.

Statistical data analysis

Normalization of the raw data and the statistical comparative analysis between the two groups (patients vs controls) was performed using the GenEx v.6 software, as suggested by the array manufacturer (Exiqon). We normalized and converted raw data into relative quantities which were log transformed in order to respect the statistical linear conditions. The differential expression of miRNAs between groups (cancer patients vs controls) was compared using the t test, using $p<0.05$ for statistical significance.

Characteristics	Cases(N=15)	Controls(N=15)
Age (years±SD)	64.9 (±5.45)	51.3 (±8.27)
PSA N (%)		
<4 ng/ml	0 (0.00)	15 (100.00)
4-10 ng/ml	6 (40.00)	0 (0.00)
=10 ng/ml	9 (60.00)	0 (0.00)
Gleason Score N (%)		
5-6	2 (14.28)	
7	10 (71.43)	
8-10	2 (14.28)	

Table 1.
Clinical and demographic characteristics of the study participants

RESULTS

The demographic and clinicopathological characteristics of the patients included in this study are shown in Table 1. The PCa patients (cases, 15) were older than the healthy subjects (controls, 15), and they had PSA levels above 4 ng/ml. The majority of the cases presented prostate tumors with a Gleason score above 7 (85.71%). All control subjects had PSA levels under 4 ng/ml.

We analyzed a panel of different miRNA species on the 384-well plates provided in the Exiqon kit. After the pre-processing steps, 140 miRNAs passed the imposed conditions and were further analyzed. Several of the 140 miRNAs analyzed presented statistical significant differences in expression levels from PCa patients' samples relative to healthy controls. With respect to the normalization methods, these differentially expressed miRNAs are presented in Table 2.

PCa vs. Controls	Spike-in normalization		Global mean normalization		NormFinder normalization	
	FC	p	FC	p	FC	p
miR-1972	-7.73	0.00028	-5.63	0.0012	-5.05	0.002
miR-590-5p	-3.35	0.0029	-2.44	0.003	-2.19	0.009
miR-655	-2.29	0.003	-1.67	0.036	-	-
miR-2110	-2.4	0.016	-	-	-	-
miR-491-5p	-2.36	0.031	-	-	-	-
miR-346	-2.06	0.032	-	-	-	-
miR-150-5p	-1.84028	0.032339141	-	-	-	-
let-7e-5p	-2.00962	0.040985808	-	-	-	-
miR-376c-3p	-2.42043	0.044122733	-	-	-	-
let-7d-3p	-	-	-1.75	0.029	-	-
miR-199a-5p	-	-	2.16	0.038	2.41	0.022
miR-15b-5p	-	-	-1.35	0.039	-	-
miR-139-5p	-	-	1.75	0.04	1.95	0.021
miR-301a-3p	-	-	2.33	0.045	2.59	0.022
let-7c	-	-	-	-	1.32	0.03
miR-30d-5p	-	-	-	-	1.77	0.039
miR-143-3p	-	-	-	-	1.82	0.04
miR-148b-3p	-	-	-	-	1.51	0.05

Table 2.

Differentially expressed miRNAs using three different normalization methods (FC-Fold Change, p – p value

Using the cel-miR-39 spike-in normalization method, as well as in the case of the NormFinder algorithm, there were 9 miRNAs differentially expressed in plasma exosomes, while using the global mean normalization, the number of differentially expressed miRNAs was 8. The Venn diagram in Figure 1 shows the dysregulated exosomal miRNAs with all three normalization strategies. Interestingly, there were only two microRNAs (miR-1972 and miR-590-5p) which were differentially expressed in patients' plasma exosomes compared to healthy controls with all three normalization methods.

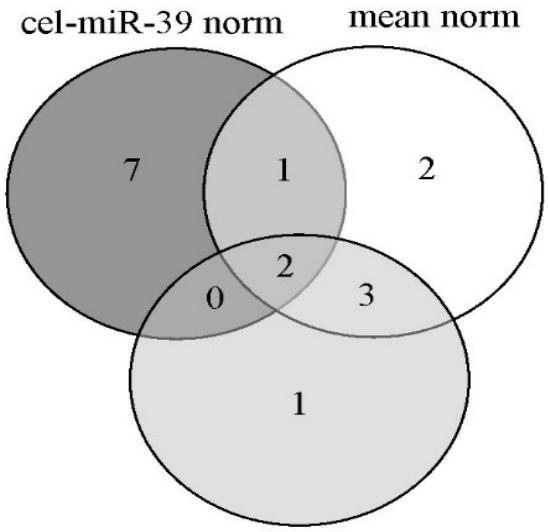

Figure 1.

Venn diagram of exosomal miRNA differential expression using three normalization methods

The Receiver Operating Characteristic (ROC) curve analysis results for miR-1972 and for miR-590-5p are presented in Figure 2. The AUC value was the same for both miRNAs (0.808), with a sensitivity and specificity value of 0.8 and 0.733 for miR-1972, and 0.933 and 0.6 for miR-590-5p, respectively.

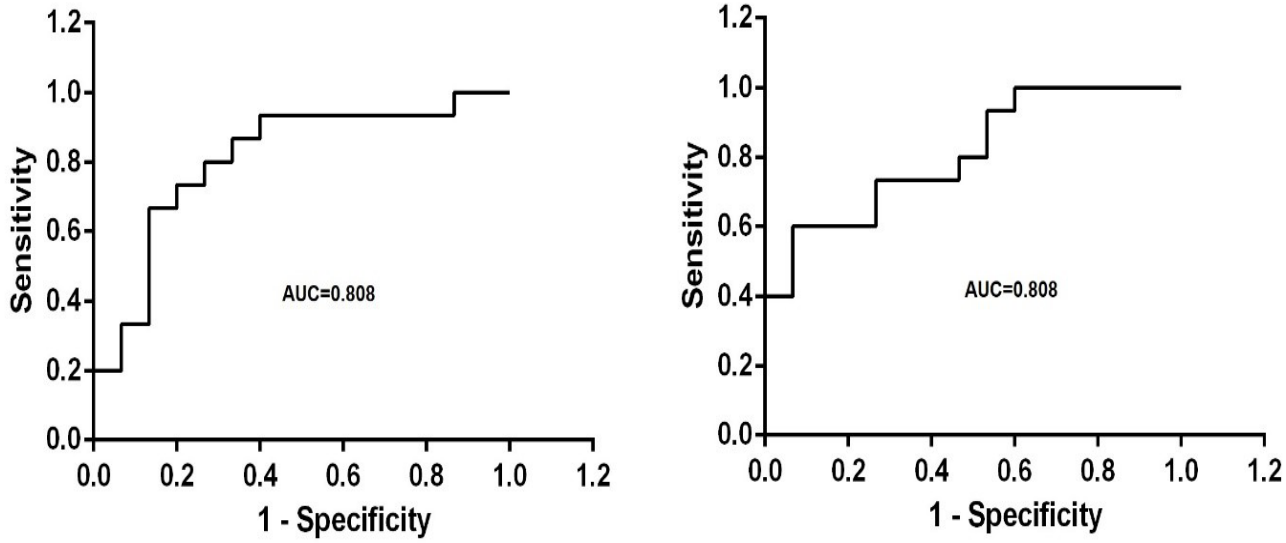

Figure 2.

ROC curve for miR-1972 (left) and miR-590-5p (right)

Furthermore, using the Ingenuity Pathway Analysis software (IPA, Qiagen) we investigated the molecule signaling networks and the biological functions for eight of these differentially expressed miRNAs (miR-1972, miR-590-5p, let-7d-3p, miR-655, miR-199a-5p, miR-15b-5p, miR-139-5p and miR-301a-3p.), and it has been shown that the most significant pathology represented is cancer (data not shown).

DISCUSSION

This study aimed to analyze and compare gene expression of a miRNA panel in PCa patients' plasma exosomes and healthy controls. From a panel of 140 miRNA species (after the pre-processing step), we found, using three different normalization methods, several miRNAs which presented statistically significant ($p<0.05$) differences in the patients' samples compared to healthy controls. Two of these miRNAs (miR-1972 and miR-590-5p) maintained a difference in expression levels in the same direction, regardless of the normalization method used.

In this study, we chose to focus on miRNAs from plasma exosomes due to a series of advantages that they possess [16-20]. Exosomes are a class of EVs produced by most eukaryotic cells. It is believed that they are involved in cell-to-cell communication, by carrying markers of cells of origin (including miRNAs). They are found in biological fluids, including blood (plasma/serum) and urine[20]. A study conducted by Cheng et al. has found that exosomal miRNAs are protected against RNase treatment, compared to circulating miRNAs, as well as that their quantity is enriched inside these small vesicles, thus turning exosomes into useful tools for miRNA profiling [19].

Our findings suggest that miR-1972 and miR-590-5p might be promising novel PCa biomarkers, a fact that has to be validated in a future targeted study. One study suggested that miR-1972 could be used in the etiology of the laryngeal squamous cell carcinoma [24]. Wang Y et al. showed that miR-1972 works together with long non-coding RNA (lncRNA) DANCR in osteosarcoma. It has been proven that DANCR suppressed ROCK1-mediated proliferation and metastasis in osteosarcoma cells, by decoying miR-1972 [25]. Another study showed that miR-1972 has 50 target genes and that it is involved in G2-M cell cycle arrest when overexpressed in KCL22 cells [26], but there is no study involving miR-1972 in PCa to date.

MiR-590-5p has been studied in various other types of cancer and other diseases. Thereby, Germano et al. have found that the overexpression of miR-590-5p in cells infected by Coxsackievirus B are more susceptible to infection, therefore turning this type of miRNA into a proviral one [21]. However, there are not many findings in literature about this type of miRNA and its role in PCa. Jalava et Al. have found that miR-590-5p was differentially expressed in CRPC compared with benign prostate hyperplasia (BPH), in a microarray analysis [27]. Nevertheless, miR-590-5p has been studied in other types of malignancies. In non-small cell lung cancer, this type of miRNA targets directly the protein-coding gene GAB1, which plays a central role in cellular growth response, transformation and apoptosis [22,23]. In addition, in breast cancer, miR-590-5p was found to play also a tumor suppressor role, by downregulating the Wnt-β-catenin signaling pathway, thus acting as an inhibitor in key points of cancer development, like migration, invasion, and epithelial-mesenchymal transition (EMT) [24]. MiR-590-5p has been also found to play a role in hepatocellular carcinoma, as a tumor suppressor. It appears to target the yes-associated protein 1 (YAP1) gene, that is involved in the chemoresistant phenotype of HCC cells [28].

There are certain limitations to our study, especially, -regarding the low number of subjects included and the fact that it is a screening study only. Future larger validation studies are needed to confirm the biomarker potential of these miRNAs in PCa.

CONCLUSIONS

We are moving towards an era of minimally-invasive, liquid biopsy approach, where biomarkers such as miRNAs could represent promising novel tools of more specialized diagnostic strategies, as well as therapeutic targets. Our preliminary results from this screening study revealed certain miRNA species that were differentially expressed in plasma exosomes of PCa patients relative to healthy controls. These findings need to be validated in future studies in order to confirm their possible biomarker potential for PCa.

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