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ORIGINAL RESEARCH

MicroRNAs as Potential Liquid Biopsy Biomarker for Patients with Castration-Resistant Prostate Cancer

Nicolas Fernandez[1], Julian Chavarriaga[2], Paola Ayala[3], Adriana Pedraza[4], John Bolivar[5], Juan Guillermo Prada[6], Juan Guillermo Cataño[6], Herney Andres García-Perdomo[7], Juliana Villanueva[6], Daniela Varela[6], Ignacio Zarante[3]

[1]Division of Urology Seattle Children's Hospital, University of Washington, Seattle, WA, USA; [2]Division of Urology, Clínica Imbanaco - Quiron Salud, Cali, Colombia; [3]Human Genetics Institute, Pontificia Universidad Javeriana, Bogota, Colombia; [4]Division of Urology Clínica Desa, Cali, Colombia; [5]Division of Urology, Department of Surgery, Hospital Universitario San Ignacio, Bogota, Colombia; [6]Division of Urology, Hospital Universitario San Ignacio, Pontificia Universidad Javeriana, Bogota, Colombia; [7]Division of Urology, Department of Surgery, Universidad del Valle, Cali, Colombia

Correspondence: Nicolas Fernandez, Division of Urology, Seattle Children's Hospital, University of Washington, 5801 Sand Point Way NE, Seattle, WA, 98105, USA, Tel +1 206 351 2618, Email nicolas.fernandez@seattlechildrens.org

Purpose: To identify micro-RNAs (miRNAs) expression profiles in peripheral blood plasma that could play a role as potential biomarkers in patients who progressed to castration-resistant prostate cancer (CRPC). Liquid biopsy analysis of miRNAs is a fast-developing field with a considerable likelihood to predict tumor progression and metastasis by targeting genes involved in oncogenesis.

Patients and Methods: Differential expression analysis of miRNAs profile in CRPC patients was performed by creating small RNA libraries of circulating miRNAs using HiSeq2500 Illumina platform. A secondary analysis of aligned reads with miRNA identification and quantification was performed using miARmaSeq. Using the Bowtie algorithm, the selected variants were compared to reference nucleotide sequence GRCh38 and miRbase. Novel miRNA sequences were structurally analyzed using mirDeep2®.

Results: A total of 16 patients with CRPC were included for analysis. Identified circulating miRNAs were hsa-miR-885-3p, hsa-miR -4467, hsa-miR-4686, hsa-miR-146a-3p, hsa-miR-6514-5p. Genes identified as regulated by these miRNAs were *GPR56, BDNF, CTNND1, C17orf62*, and *DTNA*.

Conclusion: We explored the miRNA expression profile in patients with CRPC, identifying five miRNAs implicated in the regulation of genes involved in prostate cancer (PCa) oncogenesis and progression. We also found miRNA 855–3p in peripheral blood for the first time, which has a critical role in tumor growth mechanisms and higher expression profile than in healthy individuals.

Keywords: prostatic neoplasm, prostatic neoplasms, castration-resistant, microRNAs, biomarkers, genital neoplasms, male

Introduction

Precision medicine in PCa integrates all relevant patient data to guide medical care and directs targeted therapies to improve oncological outcomes. Liquid biopsy analysis of MicroRNAs (miRNAs) is a field with a colossal future to predict tumor progression and metastasis by selecting genes involved in oncogenesis.[1–5] miRNAs are small non-coding RNAs that regulate gene expression by messenger Ribonucleic acid (mRNA) cleavage and translational suppression at the ribosome.[6,7] Bioinformatic predictions estimate that miRNAs regulate up to 30% of all protein-coding genes. miRNAs can prevent the transformation to cancer, but in the case of abnormal expression patterns, they may also promote it by targeting abnormal cellular functions in PCa.[6–8]

In biological fluids, the expression of miR-19b, miR-30e, miR-92a, miR-125b, miR-200b, miR-205, miR-378a, and miR-660 demonstrated to be associated with PCa oncogenesis. These are involved in cell cycle regulation,[9] androgen receptor (AR) signaling,[9,10] cell proliferation and differentiation,[11,12] epithelial-mesenchymal transition (EMT),[13–15] cell

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growth and apoptosis,[2,16] the extraprostatic extension of the tumors,[17] potential of metastasis,[3,13] biochemical recurrence[12] and castration-resistant prostate cancer (CRPC).[4,18]

Tissue and liquid biopsy miRNAs had been described to have targets involved in PCa oncogenesis such as FoxO, p53, p63 (miR-205) tissue growth factor-beta (TGF- β), ErbB, tumor necrosis factor (TNF), hypoxia-induced factor (HIF), mitogen-activated protein kinase (MAPK) (MiR-378), Wnt, PTEN (MiR-19b), the mTOR signaling pathway (miR-125b). It has been reported that there is a diminished expression of specific miRNAs (hsa-miR-101, −138, −186, −224, −26a, −26b, −374a, −410, −660) in PCa and is inversely correlated with up-regulation of their putative target genes such as AMACR, EZH2, PSGR, TRPM8 and PSMA.[6,7,19]

As we continue further to understand the functional roles of miRNAs in CRPC, current data suggest that miRNAs appear to have a role in the relationship of the androgen receptor (AR) and CRPC; it has been found that miRNA expression alterations occur during the development of CRPC. The development and maintenance of CRPC may be connected to the increase of miR-221/-222 owing to its involvement in essential components for AR functional integrity. miR-21 has also been found to be overexpressed in patients with CRPC, androgen Patients with chemotherapy-resistant CRPC had the highest miR-21 levels compared with those with localized PCa. Also, the investigation of miR-141 levels demonstrated a significant elevation in patients with metastatic cancer compared with those with localized disease.[33,34]

In a study trying to determine if there was an association between circulating microRNAs and docetaxel chemotherapy outcome in CRPC patients, non-responders to docetaxel and patients with shorter survival had high pre-docetaxel levels of miR-200 or decreased/unchanged post-docetaxel levels of miR-17. Additionally, miRNAs can be exploited to develop novel therapeutic modalities. For example, anti-miR125b sensitized PCa cells to cisplatin. It could be explained because miR-125b inhibition may play a role in increasing the efficacy of current therapy as p53 functionality is required for docetaxel sensitivity in PCa.[33,34]

This study aimed to identify miRNAs expression profiles in blood plasma that could be potential biomarkers in patients who progressed to CRPC.

Materials and Methods

After IRB approval, patients who fulfilled CRPC criteria who attended the outpatient clinic were offered to participate in this study at the time of diagnosis. Patient selection was established using castration-resistant criteria by the European Association of Urology.[11] All patients had to be older than 60 years of age at the diagnosis. We established a family history of PCa as an exclusion criterion. Demographic and clinical data were compiled, including time since the initial diagnosis of either localized or metastatic PCa and initial PCa staging.

We used peripheral blood samples from each participant at the time of CRPC diagnosis, including controls. Samples were centrifuged at 10.000 rpm, then plasma was extracted, and RNA was purified. miRNA extraction was performed using the kit NucleoSpin® miRNA plasma. Precise analysis of miRNAs profile in PCA patients was achieved by creating small RNA libraries using HiSeq2500 Illumina platform for sequencing 15 million reads per sample. Quality control after sequencing was done using FastQC®.[15] A secondary analysis of aligned reads with miRNA identification and quantification was performed using miARmaSeq.[16] Two healthy participants with no known PCa were included as controls for miRNA analysis. Generated low-quality reads were cleaned using the Cutadapt® program.[17] The final selected variants were compared to reference nucleotide sequence GRCh38 and miRbase using the Bowtie algorithm.[18] Novel miRNA sequences were structurally analyzed using mirDeep2® and targeted miRNA miR885-3p, which was found for the first time in peripheral blood.[19] A differential expression analysis comparing cases vs healthy controls was performed using the algorithm EdgeR.[20]

Results

Thirty-two potential investigation subjects were identified at the outpatient clinic during the study period. All of them were offered to participate in the study. After applying inclusion, exclusion criteria and evaluating the availability of blood samples in terms of enough material for sequencing, final enrollment was only possible for 16 patients. From those, five patients were initially diagnosed with metastatic hormone-sensitive prostate cancer (mHSPC), two with locally advanced disease, and seven had localized disease. The lasting two were considered healthy controls. The 14 patients

who progressed to CRPC; had a median iPSA level of 31.1 ng/mL (IQR 10.77–83.4). Six (42.8%) had an ISUP grade group 3, three patients were ISUP grade group 5, and three were group 1. Patients' characteristics are shown in Table 1.

A total of 11 miRNAs were identified in the studied population, these were: miR122-3p, miR122-5p, miR885-3p, miR1246, miR375-3p, miR455-3p, miR455-5p miR4467, miR4686, miR146a-3p, miR6514-5p. Genes identified as more regulated by this miRNA were: *GPR56, BDNF, CTNND1, C17orf62*, and *DTNA*.

In Figure 1, we can observe a heatmap showing distinct miRNA expressions in CRPC patients (X1-X7, X9-13, X15, X16) vs two healthy controls (X8 and X14). The green color indicates lower than mean intensity, and red represents higher than mean intensity. Each row represents a miRNA, and each column represents a sample.

The chosen miRNAs were miR4467, miR4686, miR146a-3p, miR6514-5p, and this turned out to be the most upregulated miRNAs compared to healthy controls. We found that miR885-3p was also upregulated, which was not described in the literature by the time of data analysis. Table 2 shows the gene targets of each of these miRNAs and their respective protein targets and known functions (Table 2).

Discussion

Liquid biomarkers are a promising new alternative for diagnosing or stratifying PCa and are currently under intensive research. Serum or urine samples are obtained by non-invasive methods and had the advantages of overcoming the limitation of multifocality and the high degree of heterogeneity in PCa tissue biopsies. The most common biomarkers in liquid biopsies are exosomes carrying miRNAs, circulating tumor DNA (ctDNA), and circulating tumor cells (CTC). Exosomes are released by all kinds of cells and are found in urine, saliva, blood, and other body fluids. When dysregulated, exosomes transfer miRNA contents contribute to oncogenesis or progression by gene up-regulation of onco-miRNAs (oncomiR) or downregulation of tumor-suppressing miRNAs. It has been shown that miRNAs are dysregulated in neoplasms, and their precursors and enzymes related to their biogenesis are also aberrantly expressed. miRNAs play three leading roles in clinical practice: diagnosis, prognosis, and therapeutic significance.

Table 1 Patients' Clinical Characteristics

Patient	TNM	Gleason Score	ISUP Grade/Group	iPSA
1	cTXNXM1B	4+5	5	86 ng/mL
2	cT3N3M0	4+5	5	91.19 ng/mL
3	cTXN0M0	4+3	3	0.067 ng/mL
4	T4N1M0	4+4	4	43.05 ng/mL
5	cTXNXM1B	4+3	3	75.87 ng/mL
6	cTXNXM1B	4+3	3	150 ng/mL
7	T2CNXM1B	4+3	3	14.4 ng/mL
8	cT2BNXM1B	4+4	4	1603 ng/mL
9	cT1CNXMX	3+3	1	8.6 ng/mL
10	cT1CNXM0	4+3	3	9.56 ng/mL
11	CT1CNOMXR0	3+4	2	5.39 ng/mL
12	CT2BNXM0	3+3	1	35.33 ng/mL
13	cT1CNXM0	4+5	5	19 ng/mL
14	cT2ANXMX	3+3	1	26.9 ng/mL

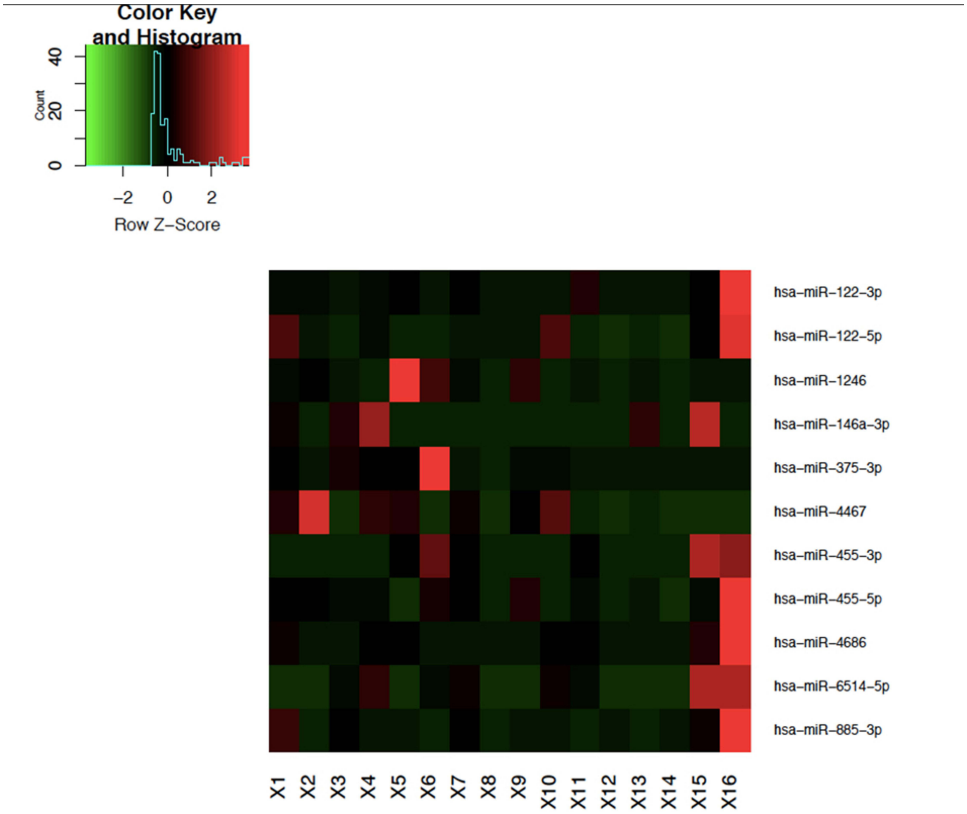

Figure 1 Heatmap showing differential miRNA expression in CRPC patients (X1-X7, X9-13, X15, X16) vs healthy control group (X8 and X14 columns). The green color indicates lower than mean intensity, and red represents higher than mean intensity (Row *Z* score). Each row represents a miRNA, and each column represents a sample.

The diagnostic utility has been widely assessed in blood and urine samples. Bryant et al compared miRNAs in plasma microvesicles of recurrent PCa patients (n=47) with non-recurrent patients (n=72). They found miR-141 and miR-375 were upregulated in patients with recurrent metastatic PCa (AUC 0.8).[21] Those results were confirmed by Li et al, which evaluated 20 patients with PCa and 20 with BPH. These authors found that plasma miR-141 is a diagnostic marker, which correlates with tumor stage and metastasis.[22]

A search of biomarkers for PCa in urine with a miRCURY LNA miRNA qPCR panel was conducted by Lekchnov et al, who performed a comparative analysis of miRNA expression in PCa patients, benign prostatic hyperplasia (BPH) patients, and healthy subjects. They finally analyzed 84 miRNA expression and found that the most accurate were miRNAs pairs were miR-107-miR-26b.5p and miR-375.3p-miR-26b.5p in the urine supernatant fraction that discriminated the group of healthy patients and PCa patients, as well as miR-31.5p-miR-16.5p, miR-31.5p-miR-200b, miR-31.5p-miR-30e.3p, and miR-31.5p-miR-660.5p in the fraction extracellular vesicles (EV) that were different between healthy men and benign prostate hyperplasia patients.[23] They ran a miRNA-based diagnostic system algorithm for PCa, discriminated 24 miRNAs of the previously mentioned 84 miRNAs, and discriminated PCa with 97.5% accuracy.[24]

The researchers, as mentioned earlier, validated a panel of 12 cell-free miRNAs in three biological fluids: urine extracellular vesicles, clarified urine and plasma from 10 PCa patients, 8 with BPH, and 11 healthy controls. Eight of the miRNAs that were found in urine vesicles revealed great promise. These miRNAs (miR-19b, miR-30e, miR-31, miR-92a, miR-125, miR-200, miR-205, and miR-660) when combined into six ratios (miR-125b/miR-30e, miR-200/miR-30e, miR-205/miR-30e, miR-31/miR-30e, miR-660/miR-30e, and miR-19b/miR-92a) were able to classify patients with PCa, BPH and healthy donors with 100% specificity and sensitivity.[25] Three more miRNA pairs discriminated PCa and HD with 100% specificity and 90% sensitivity, miR-378/miR-19, miR-425/miR-92 and miR-22/miR-19 with an AUC = 0.97,

Table 2 miRNAs and Their Respective Targets

miRNAs	Gene Targets	Protein Targets and Known Functions
miR4467	• YI • CTCF • ZNF623 • RXRA • PRDM10 • HNF4A • MAX • SPI • MYC • HNRNPL • HNF4G • GABPA	• Activate or repress the promoters. • *DNA-binding transcription factor activity* • B cell differentiation and tumor suppression • Oncoproteins implicated in cell proliferation, differentiation, and apoptosis • Immune responses, response to DNA damage, and chromatin remodeling
miR4686	• MIR4686 • TH • lnc-INS-3 • ENSG00000236710 • lnc-INS-2 • INS • NONHSAG007414.2	• The rate-limiting enzyme in the synthesis of catecholamines hence plays a key role in the physiology of adrenergic neurons • Encoding of Insulin • Multiple affiliations with the lncRNA class.
miR146a-3p	• MIR146A • lnc-ATP10B-2 • MIR3142HG • PTTG1 • ATP10B	• Some of the encoded miRNA targets are the transcripts for tumor necrosis factor, interleukin 1 receptor-associated kinase 1, interleukin 1-beta, TNF receptor-associated factor 6, and complement factor H.
miR6514-5p	• TMEM223 • MIR6514 • MIR6748 • MN297054 • lnc-TMEM223-1 • piR-55414 • BSCL2 • SNHG1 • NXF1 • CSKMT • STX5 • STX5-DT • TUT1 • TAF6L • GANAB • INTS5 • MTA2 • AHNAK • TTC9C • WDR74 • NONHSAG008544.2 • TMEM179B • lnc-TMEM223-3	• Recognizes target mRNAs through imperfect base pairing with the miRNA and most commonly results in translational inhibition or destabilization of the target mRNA
miR885-3p	• MIR885 • SEC13 • GHRLOS • LINC00852 • GHRL • HSALNG0024109 • ATP2B2	The mature miRNA is incorporated into a RNA-induced silencing complex (RISC), which recognizes target mRNAs through imperfect base pairing with the miRNA and most commonly results in translational inhibition or destabilization of the target mRNA

0.99 and 0.92 respectively.[25] They are working on independently verifying the selected miRNA ratios with bigger tissue samples from donors to reach a 95% power.

The prognostic implications of miRNAs have been described by Endzelins et al, they analyzed plasma EV samples from PCa patients (n=50, 26 GG1, and 24 GG ≥ 4) and reported that let-7a-5p from plasma EVs were lower in PCa patients with high Gleason GG ≥4 contrasted to those with Gleason GG1.[26] In contrast to these findings, Watahiki et al and Foj et al found no statistically significant differences in the let-7 profile between mCRPC and localized PCa and low-risk PCa vs High-risk PCa, respectively.[27,28] Huang et al showed that the combination of miR-1290 and miR-375 predicted OS in patients with CRPC; patients with a high level of both miRNAs had a mortality of 80% vs 10% amongst those with average plasma concentrations.[29] Bhagirath et al found plasma miR-1246 as a diagnostic marker for PCa with a specificity of 100% and a sensitivity of 75%. They also found that it could be a metastasis predictor with an AUC of 0.69.[30]

The therapeutic significance of miRNAs has also been studied, and serial miRNAs were found to be involved in docetaxel-resistant PCa and androgen deprivation therapy (ADT) responses and progression to CRPC.[31,32] Li et al reported 19 miRNAs (miR- 16, miR-203, and miR-451) upregulated and ten miRNAs (including miR-141 and miR-429) down-regulated chemoresistant PCa. Plasma miR-21 has also been reported in chemoresistant PCa.[32] Huang et al had reported that plasma miR-1290 and miR-375 were upregulated in CRPC patients. However, the article states that additional studies are required to confirm those mentioned above.[31]

A trending topic in PCa is Circulating biological materials originating from the tumor. Amongst those are the CTC which had received an FDA regulatory clearance to predict overall survival (OS) in mCRPC. Bono et al evaluated 232 patients with mCRPC and stratified them into favorable or unfavorable risk groups (<5 and ≥ 5 CTC/7.5mL) and enumerated the CTC with Cell Search system®; found that CTC predicted OS better than PSA decrement, AUC was 0.81 to 0.87, achieving statistical significance. Median OS was shorter in an unfavorable group than in (11.5 vs 21.7 months).[1] Circulating ncRNAs such as miRNAs and KLK3 a mRNA also represents a promising source of cancer- and therapy-related predictors, specifically of progression-free survival, as shown in two studies by Boerrigter and Benoit et al[35,36] Still, long-term follow-up and further validation studies are needed.

Our results explored the miRNA profile in patients with CRPC identifying five previously described miRNAs. The miRNA 885–3p has previously been described in PCa as a hypoxia response exosome that may correlate to tumor progression.[30–34] It has been shown higher miRNA 885 levels in patients with PCa than healthy individuals. To our knowledge, this is the first time such a miRNA has been reported in circulating peripheral blood in CRPC patients. Further studies may be needed to explore this as a potential biomarker for PCa clinical progression.

We acknowledge the limitations of being a small sample of patients. Nonetheless, the presence of circulating miRNAs identified and associated with gene interactions confirms that it might be feasible to use this approach as a potential prognostic tool for CRPC patients. Furthermore, validation by RT-qPCR of the reported miRNAs should be performed in a new validation cohort of samples and healthy controls, considering the small sample size of our study. Also, we recognized this was a pilot study that aimed to determine circulating miRNAs in patients with CRPC in the Latin-American setting, which has not been widely evaluated previously. In the future, we hope to continue analyzing miRNAs, and in other stages of PCa, including mHSPC, Biochemical recurrence and including BPH patients as healthy donors.

Conclusions

Emerging PCa molecular biomarkers such as miRNAs carry a great potential to revolutionize the diagnosis and treatment of this and other cancers. Our study explored the miRNA expression profile in 46 patients with CRPC, identifying eleven miRNAs, of which five are involved in regulating genes in PCa oncogenesis and progression. We also found miRNA 855–3p in peripheral blood for the first time, which has a critical function in the mechanisms of development of the tumor and higher expression profile than in healthy individuals. Further investigation of these miRNAs in the future is undoubtedly warranted. In other disease settings, especially mHSPC and biochemical recurrence, and for other miRNAs, additional prospective large studies are needed to confirm their potential as tumor markers and better understand the limitations of these novel serum markers.

Abbreviations

PCa, prostate cancer; CRPC, castration-resistant prostate cancer; BPH, benign prostatic hyperplasia; OS, overall survival; ADT, androgen deprivation therapy; mHSPC, metastatic hormone-sensitive prostate cancer; CTC, circulating tumor cells.

Data Sharing Statement

The datasets used are available upon request.

Ethical Approval

We did this investigation after obtaining informed consent from the patients and prior authorization from the ethics committee of Hospital San Ignacio. IRB number 215/148. It adheres to the regulations related to the management of clinical information on patients and the Declaration of Helsinki of 1975, revised in 2013. Likewise, considering Article number 11. Resolution 8430 of 1993 of the Colombian jurisdictions classified this investigation as "Investigation without risk".

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

The authors declare that they are responsible for all aspects of the work. They ensure that the questions related to their study's accuracy or integrity are appropriately investigated and resolved.

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