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The role of miRNAs as biomarkers in prostate cancer

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ABSTRACT

There is an urged need of non-invasive biomarkers for the implementation of precision medicine. These biomarkers are required to these days for improving prostate cancer (PCa) screening, treatment or stratification in current clinical strategies. There are several commercial kits (Oncotype DX genomic prostate score®, Prolaris®, among others) that use genomic changes, rearrangement or even non-coding RNA events. However, none of them are currently used in the routine clinical practice. Many recent studies indicate that miRNAs are relevant molecules (small single-stranded non-coding RNAs that regulate gene expression of more than 30% of human genes) to be implement non-invasive biomarkers. However, contrasting to others tumors, such as breast cancer where miR-21 seems to be consistently upregulated; PCa data are controversial. Here we reported an extended revision about the role of miRNAs in PCa including data of AR signaling, cell cycle, EMT process, CSCs regulation and even the role of miRNAs as PCa diagnostic, prognostic and predictive tool. It is known that current biomedical research uses big-data analysis like Next Generation Sequencing (NGS) analysis. We also conducted an extensive online search, including the main platforms and kits for miRNAs massive analysis (like MiSeq, Nextseq 550, or Ion S5™ systems) indicating their pros, cons and including pre-analytical and analytical issues of miRNA studies.

1. Introduction

Prostate cancer (PCa) is the second most common cancer among men, with a rate of 1.1 million of new cases diagnosed in 2012 [1]. There are several clinical criteria used as screening or stratification parameters such as prostate specific antigen (PSA) value, imaging diagnostics and histopathological scores (e.g. Gleason score), however these ones are not efficient as prognosis biomarkers. Currently there are some prognostic commercial biomarkers, such as Oncotype DX genomic prostate score® (Genomic health, CA, USA), Prolaris® (Myriad Genetics Inc., UT, USA), ProMark® (Metamark, MA, USA), ExoDx™ Prostate (IntelliScore, MA, USA) or Decipher® (GenomeDX, Vancouver, Canada), among others, all based on several SNPs panels or epigenetic modifications; but none of them show accurate data [2]. Some molecular biomarkers are in use, such as prostate cancer gene 3 (PCA3 or DD3) detected in urine and based on specific PCa tissue non-coding RNA, and which is currently included in the European Association of Urology (EAU) guidelines as a factor for biopsy decision making. TMPRSS2:ERG fusion transcripts are also reported as urine biomarkers that seems to be indicative of PCa aggressiveness upon biopsy. Mainly PCA3 but also TMPRSS2:ERG are considered with a valuable potential in PSA quandary situations. It is also suggested that the combination of PCA3

Abbreviations: ADT, androgen deprivation therapy; AR, androgen receptor; BCR, biochemical recurrence; BPH, benign prostate hyperplasia; CRPC, castration resistant prostate cancer; CSCs, cancer stem cells; dPCR, digital PCR; EMT, epithelial–mesenchymal transition; EVs, extracellular vesicles; HD, healthy donors; miRNAs, microRNAs; mPCa, metastatic prostate cancer; NGS, next generation sequencing; OS, overall survival; PCa, prostate cancer; PSA, prostate specific antigen; qPCR, real-time PCR; RP, radical prostatectomy; sRNA, small RNA

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with TMPRSS2:ERG fusion transcripts could improve the specificity and sensitivity compared to the single use of these both non-invasive markers [3].

The main aim of current medicine is to develop efficient and non-invasive biomarkers. Most of promising PCa biomarkers are developed in urine, such as ExoDx™ Prostate (Intelliscore, MA, USA) which uses exosomal RNA for reducing the rate of unnecessary biopsies [4]; or previous described PCA3 and TMPRSS2:ERG. But it is true that there are also a high proportion of biomarkers used in tissue such as Oncotype DX genomic prostate score® (Genomic health, CA, USA), Prolaris® (Myriad Genetics Inc., UT, USA), and ProMark® (Metamark, MA, USA); used for monitoring the aggressiveness of the tumor. Moreover Decipher® (GenomeDX, Vancouver, Canada) is also detected in tissue sample but mainly focused in monitoring treatment course after prostate surgery. See more details in Supplementary Table 1.

Concerning miRNAs, miRview™mets (Rosetta Genomics, PA, USA) was the first miRNA-based cancer diagnostic available test. This test studies a 48-panel of miRNAs in tissue for 22 tumor types to determine the origin of metastasis. A second-generation test (miRview mets^2), increases the number of tumor types to 49 based in a 64 miRNAs panel [5]. However, up to now there is any test based on miRNAs for the diagnosis or prognosis of primary PCa.

The development of advanced high-throughput 'omics' technologies has enabled the use of novel and more efficient markers guiding the prognosis and improving PCa treatment´s strategies. Currently, there are numerous molecular markers used with this aim, mainly focused on the mechanisms of castration resistance, trying to offer the best treatment opportunities. The main molecular markers are focused on: i) androgen receptor (AR) mutations, overexpression, or splice variants; ii) CYP17 variants; or iii) PARP (Poly (ADP-ribose) Polymerases) inhibitors which are the molecular targets of several treatments such as bicalutamide, flutamide, enzalutamide, abiraterone, docetaxel or cabazitaxel [6].

Among future predictive biomarkers those ones based on liquid biopsy components such as circulating tumor cells (CTCs) or free nucleic acids should also be mentioned. Here, we are focusing on the role of miRNAs, mainly in their use as cancer biomarkers. It is well known the role of miRNAs modulating important cellular cancer processes/pathways and their stability in body fluids; they are points that make these molecules as promising biomarkers.

Moreover, there are data suggesting their diagnostic (miR-21and miR-141) [7] and prognosis role (miR-141 and miR-375) in PCa [8]. All previous mentioned miRNAs characteristics suggest that these biomolecules could improve the current limitations existing in commercial panels; mainly due to their broad molecular activities and their non-invasiveness option of study. The goal of the present review is to determine the most important roles and limitations of miRNAs as biomarkers for PCa in current medicine.

2. MiRNAs

MicroRNAs (miRNAs) are small single-stranded non-coding RNAs of 18–25 nucleotides in length. These molecules regulate the expression of more than 30% of human genes by binding to the 3′-untranslated regions (3′-UTR) of specific mRNAs [9].

They are secreted by cells in different body fluids such as blood, urine, tears, saliva, breast milk and seminal fluid, etc. In the body fluids, miRNAs are protected to enzymatic degradation by their union with Argonaute2 protein and with high density lipoprotein (HDL); but also through their inclusion in extracellular vesicles (EVs). Their high stability in biological samples, resistance to storage conditions and easy accessibility, makes miRNAs as potential biomarkers [10].

It is known significant role of miRNAs in development and progression of several cancers by exerting important effects in oncogenes, suppressor genes, etc [11]. Their wide activity in many cancer aspects such as invasion, angiogenesis, metastasis, including the immune homeostasis and normal function of innate and adaptive immune response, among others; make relevant to reinforce their role as tumor biomarkers [12,13].

Furthermore, there are findings also supporting the therapeutic capability of miRNAs like miR-200 family inhibiting the ovarian metastasis and potentially in other cancer types. This strategy is mainly due to the unique miRNAs ability targeting a variability of cancer phenotype genes. This is the reason why identifying these small regulatory RNAs is emerging as a potential significant strategy for cancer therapeutics in the next future [14]. The current fact that some miRNAs replacement therapies; such as restoring tumor suppressor miRNAs function in tumor cells by synthetic miRNA mimics; or miRNAs expressions plasmids; are currently used in clinical trials demonstrates the great potential of this approaching to treat cancer [15].

2.1. Role of miRNAs in PCa

miRNAs play a critical role in PCa pathogenesis by regulating the expression of genes involved in AR signaling pathway; cell cycle; apoptosis; epithelial–mesenchymal transition (EMT); and metastasis; as well as in the control of stemness in cancer stem cells (CSCs) [16] (Table 1 and Fig. 1).

2.1.1. AR signaling

PCa is an androgen-dependent disease and AR signaling is directly implicated in tumorigenesis and disease progression [17]. Several miRNAs are involved in AR signaling and their interaction is relevant in the progression towards castration resistant prostate cancer (CRPC), a deadly phase of the disease [18]. Both, miR-21 and 32, have been found overexpressed in CRPC compared with benign prostate hyperplasia (BPH) [19]. miR-21 is frequently overexpressed in solid tumors including PCa, being up-regulated by AR. This miRNA promotes cell proliferation and invasion by targets and inhibit the expression of PTEN and PDCD4 genes [20,21]. Similarly, miR-32 is a target of AR that acts as an oncogene by repressing BTG2 gene. BTG2 controls cell cycle and apoptosis process [19].

2.1.2. Cell cycle and apoptosis

Several miRNAs are implicated in cell cycle deregulation through the interaction with cell cycle checkpoint genes; including clusters miR-15a/16 and miR-221/222, let-7a, miR-24 and miR-31.

The cluster miR-15a/miR-16 is located at chromosomal region 13q14 and it is frequently decreased in advanced PCa. It has a suppressive function in PCa targeting CCND1 gene which is involved in G1/S transition. In addition, miR-15a/miR-16 inhibits the expression of WNT3A, and BCL2 genes which are involved in cell proliferation and apoptosis resistance [22].

The additional cluster miR-221/222 acts as an oncogene and promotes cell cycle progression and proliferation in aggressive PC3 cells by the repression of cyclin-dependent kinase inhibitor p27^{Kip1}. However, the miR-221/-222 expression is repressed in some PCa clinical analysis [23,24].

Another important cell cycle miRNA is let-7a. Let-7a belongs to the tumor suppressor family let-7 and it is repressed in PCa tissue and cells lines. Dong and colleagues evidenced that let-7a targets and down-regulates E2F2 and CCND2 resulting in a cell cycle arrests at the G1/S phase in PC3 and LNCaP cells. Moreover, they confirmed the capability of let-7a to inhibit prostate tumor development *in vivo* in xenograft models [25].

On the other hand, an overexpression of miR-125b stimulates tumor growth by silencing pro-apoptotic genes P53, PUMA, and BAK1 [26].

2.1.3. EMT process

During EMT process, epithelial cells lose their epithelial properties (cell-cell adhesion and polarity) acquiring mesenchymal characteristics that promotes invasion and metastasis [27]. miR-205 and miR-200

Table 1
Role of some miRNAs in PCa.

miRNAs	Up (↑)/Down(↓)	Target Genes	Biological Process	References
let-7a	↓	E2F2, CCND2	Cell cycle	[25]
let-7c	↓	C-MYC	AR signaling	[35]
miR-1	↓	E2F5, CDK14, SLUG	Cell cycle, EMT	[36,37]
miR-15a/16	↓	CCND1, WNT3A, BCL2	Cell cycle, Apoptosis	[22]
miR-21	↑	PTEN, PDCD4	Cell cycle, Apoptosis, AR signaling	[20,21]
miR-24	↓↑	CDKN1B, CDKN2A, FAF1	Cell cycle, Apoptosis	[38,39]
miR-26a	↓	LIN28B, ZCCHC11,	Metastasis	[40]
miR-31	↓	AR, E2F1, E2F2, EXO1, FOXM1, MCM2	AR signaling, Cell cycle	[41]
miR-32	↑	BTG2	Cell cycle, Apoptosis	[19]
miR-34a	↓	CD44, STMN1	CSCs, Metastasis	[33,42]
miR-34b	↓	AKT	Cell cycle	[43]
miR-96	↑	FOXO1, MTSS1	Cell cycle, Metastasis	[44,45]
miR-99a	↓	NCAPG, SMARCA5, FGFR3	Cell cycle, Cell proliferation	[46,47,48]
miR-124	↓	AR	AR signaling	[49]
miR-125b	↑	P53, BBC3, BAK1	Apoptosis	[26]
miR-126	↓	ADAM9	EMT, Metastasis	[50]
miR-133b	↓	FAIM	Apoptosis	[51]
miR-141	↑↓	NR0B2, CD44, EZH2, Rho GTPases	AR signaling, CSCs, Metastasis	[52,53]
miR-143/145	↓	PROM1, CD44, OCT4, C-MYC, KLF4, ZEB2, AR	CSCs, EMT, AR signaling	[34,54,55]
miR-182	↑	ARRDC3, FOXO1	AR signaling, Metastasis, Cell cycle	[56]
miR-185	↓	BRD8 ISO2, SREBP-1, SREBP-2	AR signaling, Apoptosis	[57,58]
miR-200 Family	↓	ZEB1, ZEB2, PDGF-D, SLUG	EMT	[37,59]
miR-203	↓	MAP2K1, RAP1A	Cell proliferation, Metastasis	[60,61]
miR-205	↓	c-SRC, BCL2, AR, ZEB2, PKCε	Cell proliferation, Apoptosis, AR signaling, EMT	[62,63,64]
miR-221/222	↑	CDKN1B	Cell cycle	[65]
miR-320	↓	CTNNB1	CSCs	[66]
miR-375	↑	SEC23A, YAP1	Cell proliferation, EMT	[31,67]
miR-409	↑	RSU1, STAG2, NPRL2	EMT, Metastasis	[68]
miR-449	↓	HDAC-1	Cell cycle	[69]
miR-940	↓	MIEN1	EMT	[70]

Abbreviations: ADAM9 (ADAM metallopeptidase domain 9); AKT (AKT serine/threonine kinase 1); AR (androgen receptor); ARRDC3 (arrestin domain containing 3); BAK1 (BCL2 antagonist/killer 1); BBC3 (BCL2 binding component 3); BCL2 (BCL2 apoptosis regulator); BRD8 ISO2 (bromodomain containing 8 isoform 2); BTG2 (BTG anti-proliferation factor 2); CCND1 (cyclin D1); CCND2 (cyclin D2); CD44 (CD44 Molecule (Indian Blood Group)); CDK14 (cyclin dependent kinase 14); CDKN1B (cyclin dependent kinase inhibitor 1B); CDKN2A (cyclin dependent kinase inhibitor 2A); c-MYC (MYC proto-oncogene); c-SRC (SRC proto-oncogene, non-receptor tyrosine kinase); CTNNB1(catenin beta 1); CSCs (cancer stem cells); E2F1 (E2F Transcription Factor 1); E2F2 (E2F transcription factor 2); E2F5 (E2F transcription factor 5); EMT (epithelial- mesenchymal transition); EZH2 (enhancer of zeste 2 polycomb repressive complex 2 subunit); FAF1 (Fas associated factor 1); FAIM (Fas apoptotic inhibitory molecule); FGFR3 (fibroblast growth factor receptor 3); FOXM1(forkhead box M1); FOXO1 (forkhead box O1); HDAC-1 (histone deacetylase 1); KLF4 (Kruppel like factor 4); LIN28B (lin-28 homolog B); MCM2 (minichromosome maintenance complex component 2); MEK1 (mitogen-activated protein kinase kinase 1); MIEN1 (migration and invasion enhancer 1); MTSS1(MTSS I-BAR domain containing 1); NCAPG (non-SMC condensin I complex subunit G); NPRL2 (NPR2 like, GATOR1 complex subunit); NR0B2 (nuclear receptor subfamily 0 group B member 2); OCT4 (organic cation/carnitine transporter4); P53 (tumor protein p53); PDCD4 (programmed cell death 4); PDGFD (platelet derived growth factor D); PKCε (protein kinase C epsilon); PROM1 (prominin 1); PTEN (phosphatase and tensin homolog); RAP1A (RAP1A, member of RAS oncogene family); SLUG (snail family transcriptional repressor 2); SMARCA5 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5); RSU1(Ras suppressor protein 1); SEC23A (Sec23 homolog A, coat complex II component); SREBP-1 (sterol regulatory element binding transcription factor 1); SREBP-2 (sterol regulatory element binding transcription factor 2); STAG2 (stromal antigen 2); STMN1 (stathmin 1); WNT3A (Wnt family member 3A); YAP1 (Yes associated protein 1); ZEB1 (zinc finger E-box binding homeobox 1); ZEB2 (zinc finger E-box binding homeobox 2); ZCCHC11 (Zinc Finger CCHC Domain-Containing Protein 11).

family members (specific clusters miR-200a/ miR-200b/ miR-429 and miR-200c/miR-141) can revert EMT by targeting ZEB1 and ZEB2, both repressors of E-cadherine epithelial marker [28]. miR-205 expression is down-regulated during PCa progression [29]. But in the case of miR-200 family, several data report elevated levels of miR-200 family members in advanced PCa [8,30]. Interestingly, ZEB1 has been identified as a transcriptional repressor of miR-375 during EMT; and YAP1 (oncogene that drives EMT) as a target of miR-375 [31]. Nevertheless, overexpression of miR-375 is correlated with PCa progression and CRPC [8,30].

2.1.4. CSCs regulation

CSCs are considered a small subset population of cancer cells with stem-like properties such as self-renewal, differentiation capacity, resistance to treatments which are involved in tumor progression and metastasis [32]. Tumor suppressor miR-34a (a p53 target) is an example of a miRNA that regulates CSCs in prostate tumors. Liu et al. [33] found that miR-34a directly suppressed CD44 expression, a CSCs marker. In the same way, the cluster miR-143/ miR-145 suppresses CD44 and others CSCs markers in PCA3 including CD133, CD44, c-Myc and Klf4 [34].

2.2. miRNAs as PCa diagnostic, prognostic and predictive biomarkers

Considering that different miRNAs expression patterns seem to reflect the presence and evolution of PCa, the estimation of miRNAs profile may be useful for improving PCa management.

2.2.1. miRNAs in tissue

Several miRNAs have been evaluated in tissue as PCa diagnostic, prognostic and predictive biomarker. Wach and colleagues identified a miRNA PCa profile including miR-375, miR-143 and miR-145 able to discriminate between tumor and normal prostate tissue. The combination of this three miRNAs, could correctly classify almost 78% of tumor samples [71]. Das et al. [72] have found that high miR-1207 expression patterns correlates with clinicopathological variables like Gleason score, clinical stage, or PSA level; as well as with a higher risk for developing PCa relapses. miR-133b and miR-1 levels have been found significantly down-regulated in recurrent PCa samples in comparison to non-recurrent PCa ones after radical prostatectomy (RP) [73,74]. In

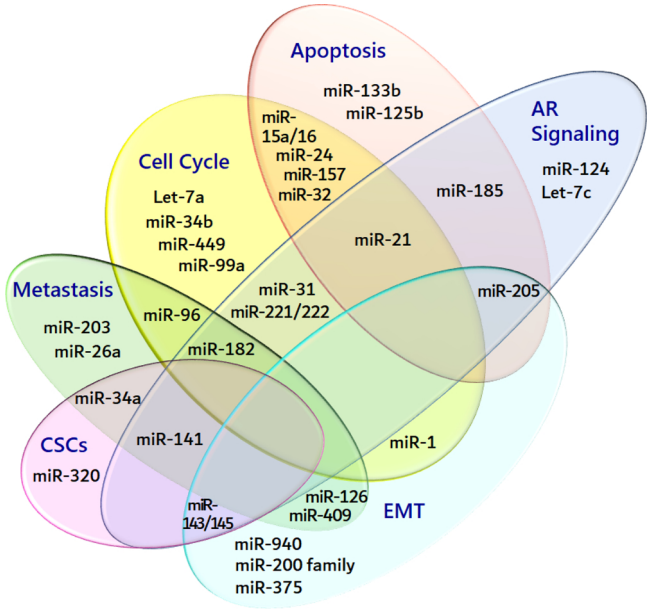

Fig. 1. Representation of role of some miRNAs in PCa.
Footnote Fig. 1. As we can see in Venn diagram of Fig. 1, whith miRNAs that have previously reported in Table 1; we have summarized main miRNAs functions. Although of the 33 studied miRNAs, 14 miRNAs have a single function, the remaining ones (57%) have two or even three functions (which makes representation in sets difficult). In this figure we distinguish 6 functions: androgen receptor signaling (AR signaling), cell cycle, metastasis, apoptosis, epithelial-mesenchymal transition (EMT) and cancer stem cells (CSCs).

addition, down-regulated miR-30c in prostatectomy tissue samples has been correlated with higher Gleason scores, an advanced pathological stages and biochemical recurrence (BCR) after surgery in PCa patients [75]. With respect to hormonal therapy, a high miR-21 expression has been associated with a shorter progression-free survival in advanced PCa patients receiving androgen deprivation therapy (ADT) [76].

2.2.2. Circulating miRNAs

Apart from tissue-based studies; the discovery of circulating miRNAs in body fluid like blood, urine or semen; offers the opportunity to identify non-invasive biomarkers [16].

In 2008, Mitchell and colleagues demonstrated for the first time PCa tumor miRNAs released in blood [77]. Since then, several studies have showed the ability of blood circulating miRNAs to distinguish PCa patients from those with other prostate pathologies, or even from healthy patients, or predicting PCa aggressiveness. Nevertheless, miR-21, miR-141 and miR-375 are the most recurrent circulating miRNAs in PCa studies [16].

Gao and coworkers showed that miR-21 and miR-375 expression patterns were increased in PCa patients and discriminate PCa patients from BPH subjects at early stages [78]. It was recently confirmed, the overexpression of miR-21 and miR-375 in PCa patients serum respect healthy donors [7]. Also, this study showed that the addition of miR-141 to previous miRNAs improved the positive predictive detection of PCa. Conversely, in a previous research carried out by Kachakova and colleagues, miR-375 expression was down-regulated in PCa patients serum samples compared to BPH controls [79].

Other potential non-invasive biomarkers proposed for PCa diagnosis are miR-139-5p [80] and miR-320 family members (miR-320a, -b and –c) [81]. Their expression in peripheral whole blood and serum, respectively, differ significantly between PCa patients, BPH patients and healthy donors (HD). Remarkably, in the study of miR-320a, -b and –c in serum, BPH patients showed the highest median expression levels while HD showed the lowest levels; being PCa patient in an intermediate level [81].

In addition, the potential role of miR-141 and miR-375 as prognostic non-invasive biomarkers has been corroborated in several investigations. Elevated miR-141 and miR-375 levels in serum of metastatic PCa (mPCa) patients have been found when comparing with non-metastatic ones [8]. Also, higher levels of these miRNAs have been correlated with higher Gleason scores or lymph-node positive status [8].

Circulating miRNAs expression profile may also be useful in PCa stratification such as miR-200c, miR-605, miR-135a*, miR-433, and miR-106a that can identified PCa aggressive forms with a 89% accuracy [82] in serum; and prediction therapies outcomes like, low circulating miR-128 [83] and miR-129 [84] expression patterns in serum and peripheral blood mononuclear cells, related with shorter BCR-free survival after RP. Otherwise, despite miR-16 and miR-195 are considered tumor suppressors in PCa [22,85], high plasma levels of miR-16, miR-195 and, miR-148a have been associated with higher Gleason scores. And elevated patterns of miR-195 with a higher risk for recurrence after surgery [86].

On the other hand, urine has also become as another important source of non-invasive biomarker for PCa and other urologic pathologies [87]. The proximity of prostate to the urethra makes the presence of exfoliated epithelial cells from prostate, urinary tract and other secreted products in the urine sediment possible, especially after a prostate massage [88].

Nevertheless, studies concerning miRNAs expression patterns in urine are limited [87]. Lately, Foj et al. reported a case-control study where miR-21, miR-141, and miR-375 were higher in PCa patients urinary pellets than in healthy subjects. Moreover, miR-141 was correlated with Gleason score; miR-21, miR-141, and miR-214 were deregulated in intermediate/high-risk PCa versus low-risk/healthy subjects [89]. Additional potential urinary miRNAs biomarkers suggested for PCa diagnosis are miR-484,miR-1825 [90], miR-222-3p*, miR-24-3p and miR-30c-5p [91]. According to Fredsøe and coworkers; the miR-222-3p*miR-24-3p/miR-30c-5p model discriminates PCa from BPH patients [91].

The urinary high miR-100/miR-200b score has been significantly associated with PCa presence; and their inclusion in a basal prediction model (digital rectal examination, age, PSA and % free PSA) enhances the predictive value regarding the prostate biopsy outcome for patients with PSA levels in the “gray area” (4–10 ng/ml) [92]. This is especially important for avoiding unnecessary biopsies, because only about 30% of men with “gray area” PSA levels have PCa [93].

2.2.3. RNA-targeted therapeutics

miRNAs mediate drug response [94] suggesting that manipulating their expression could be useful in cancer therapy. Nowadays, there are different strategies for modulating miRNAs expression; including small molecule inhibitors of miRNAs biogenesis; miRNAs vectors, miRNAs mimics; miRNAs sponges; CRISPR technology, anti-miRs; and miR-mask oligonucleotides [95].

Pre-clinical studies have demonstrated the potential role of miRNAs in PCa radiotherapy. For instance, miR-124 and miR-144 can reduce hypoxia-induced autophagy and enhance radiation sensitivity of PCa cells via reducing PIM1 [96]. Autophagy induced by hypoxia is a well-known mechanism that promotes radioresistance [96]. Similarly, ectopic expression of miR-205 inhibits autophagy and reduces PCa cells radioresistance by targeting TP53INP1 [97].

On the other hand, chemotherapy (based in taxanes) is indicated for CRPC phase [98]. miRNAs have been proposed as one of the mechanisms of resistance to taxanes. Restoring miR-199a expression increases paclitaxel sensitivity in PCa cell lines and mouse xenografts by targeting YES1, a gene implicated in cell proliferation and apoptosis resistance [99]. miR-130a, [100], miR-217, miR-181b-5p [101] and miR-451 [102], are also seem to be involved in resistance to taxanes. Re-establishing the expression of those miRNAs enhances the sensitivity to placlitaxel, cabazitaxel and docetaxel in PCa cell lines.

3. Exosomes

Exosomes are small nano-sized (30–100 nm) extracellular vesicles (EVs) secreted by almost all cells in the human body. They are present in diverse body fluids, including blood [103], urine [104], semen [105], saliva [106], among others. Due to their endosomal origin these vesicles carry proteins, lipids, and nucleic acids (including DNA and miRNAs) [107,108].

The main benefits of studying exosome-derived miRNAs are: (i) the encapsulation of miRNAs in exosomes stabilize them in body fluids, protecting from RNases, proteases and immune attack, and also making miRNAs biologically active for recipient cells [109]; (ii) exosomes have shown miRNAs enrichment compared to cells [110]. Moreover, it has been proved by Parolini et al. that tumor cells release higher amounts of exosomes than non-malignant cells because of acidic pH in tumor microenvironment [119]. That is why, exosome-derived miRNAs have emerged as new biomarkers for cancer diagnosis, prognosis and monitoring in almost all tumor types [111,112].

Studying exosome encapsulated miRNAs requires isolation methods: ultracentrifugation, precipitation, ultrafiltration, capture with antibody coated-beadsmicrofluidic [113] and quantification methods : electron microscopy, Nanosight or Western blot [114].

3.1. ExomiRNAs in PCa

In the last decade, several publications have highlighted the role of extracellular miRNAs in diagnosis and prognosis of urological tumors [115,116]. Here, we summarize recent publications of exosome derived miRNAs utility in PCa (Table 2). Peripheral blood (plasma or serum), is the leading sample for exosome isolation; however due to urological tumors characteristics urine and semen are important sources of exosomes for clinical applications.

Byant et al., found that miR-141 was highly expressed for mPCa patients in exosomes from different sources (serum, plasma and urine) being a suitable non-invasive diagnostic marker for micro-metastasic disease [117]. According to these results, Li and co-workers showed that serum levels of exomiR-141 were higher in PCa than HD and BPH patients; moreover these levels were increased in mPCa population [118].

A study performed in aggressive PCa patients revealed that exomiR-1246 expression was correlated with an increased pathological grade, positive metastasis, and poor prognosis [119]. CRPC patients have also been object of exomiRNAs study, Huang et al. found that exosomal miR-1290 and miR-375 had the potential of predicting the prognosis in those patients [120].

Significant upregulation of miR-21, miR-141, and miR-375 was found comparing PCa patients with HD in urinary pellets; while miR-214 was found significantly down-regulated. Regarding urinary exosomes, miR-21 and miR-375 were also significantly up-regulated in PCa but no differences were found for miR-141. Significant differences were found for let-7c in PCa urinary exosomes while no differences were observed in urinary pellets [89].

According to Bryzgunova et al. [121] miR-19b is a promising biomarker for PCa detection in urinary exosomes. Using lectin-based exosome agglutination method, Samsonov et al. [122] showed that miR-21, miR-141, and miR-574 were up-regulated in PCa patients compared with HD.

Extremely high concentration of EVs (including exosomes) has been reported in semen [107,123]. Moreover next generation sequencing of RNA content in semen exosomes showed that miRNAs represent around a 20% of the reads in 40–100 nt fraction. Analysis of exomiRNAs from semen could also be helpful for new biomarkers discovery in PCa and testicular carcinoma [124].

3.2. Exosomes in cancer therapy

Since exosomes are efficient EVs in cell communication by delivering molecules, they have been proposed as carriers of therapy molecules including anti-tumoral miRNAs. Drug administration using exosomes have shown an increase in cytotoxic effect of Placlitaxel in PCa cells compared to free drug [125]. This technique also show a reduction of host immune responses due to exosome manufacturing as of cells derived from each patient [126,127].

Main efforts to develop effective and safe new therapies based exosomes are focus on improving methods for loading therapeutic molecules into exosomes and their uptaking by target cells. Electroporation, transfection, cell activation or incubation are common methods for drug encapsulation; being electroporation the most used technology for miRNAs. Moreover, cell modification can be done to produce targeted exosomes with molecules that induce tropism to a cellular type [128]. GE11 and EGF- targeting exosomes were built to deliver let-7a miRNA to the EGFR-expressing tumor in a mouse xenograft model [129].

4. Methodologies for detection

Several methods can be used for miRNAs´ characterization, being the most common one, the real-time PCR (qPCR), which is usually used for monitoring individual miRNAs levels. To study the whole miRNAs set NGS technologies (miRNA profile by next generation sequencing) have grown achieving improved miRNAs profiles by the use of microarrays. There are several problems for selecting miRNAs as new and efficient biomarkers in PCa. First of all, microarrays detection, digital PCR (dPCR) or qPCR are restricted for certain probes, some examples, for the current strategies of miRNAs´ detection using arrays, can be seen in Table 3. Thus, these technologies are generally suitable for comparing expression of previously annotated miRNAs, but cannot reveal novel miRNAs. Therefore, we are going to focus on NGS technology mainly because NGS assures discovering nearly any kind of small RNA (sRNA) marker. This technology is ideal for carrying out the analysis of short nucleotide chains, such in the case of miRNAs; moreover miRNAs contain well-defined sequences; which are also crucial for efficient bioinformatic studies. But we should include several considerations before starting any miRNAs analysis: 1) Which kit does offer the best set of supplies to perform library preparation?; 2) What is the best equipment to make libraries´ sequencing? [130].

There are already many kits available for preparing sRNA libraries, but these typically only allow the simultaneous multiplexing of 48 samples, which is a problem to perform sequencing in medium and high throughput equipment [131]. Some of the most used libraries preparation kits are TruSeq™ Small RNA Library (Illumina, CA, USA), NEBNext® kit (New England Biolabs, MA, USA), NEXTflex® V2 kit (BIOO Scientific, TX, USA) and the novel SMARTer™ (Clontech, CA, USA). Moreover, the multiplexing of the samples causes another difficulty, which is the normalization. After finishing the library, it is difficult to obtain a homogeneous number of readings per sample, especially in samples with low amount of starting RNA. It is recommended for having external normalization standard that all samples should have similar starting RNA amounts and include spike-in controls. Spike-in (non-natural miRNAs those with no 5′ phosphate) are not detected by NGS (library kits that use ligation-based methodology) but non-natural miRNAs could be used for qPCR validation test. So, we have to consult normalization options with the “library kit” provider. However, the most common protocol is using miRNAs as normalizers or housekeepings. miR-16 and miR-1228 has been described as stable endogenous control for PCa lines, biopsies and circulating miRNAs, although U6 and U48 seems to be the best option for PCa miRNA normalization [132,133].

NGS and microarray data normalization of NGS and microarray data is based on the use of a factor resulting from the expression of multiple

Table 2
ExomiRNAs as diagnostic and prognostic biomarkers for PCa.

Cancer location	Biological sample	Study population	Clinical application	Results	Reference
Prostate	Plasma (Exosomes) Serum (Exosomes) Urine (Exosomes)	PCa(n = 51) mPCa (n = 15) HD (n = 28) Patients after RP: Non-recurrent (n = 72) Recurrent (n = 47) PCa (n = 70) mPCa (n = 48) HD (n = 17)	Diagnosis	Taken together results from all cohorts, **miR-141** was significantly higher in metastatic patients suggesting that **miR-141** testing could be clinically useful identifying micro-metastatic disease. Moreover, **miR-200b** and **miR-375** were highly expressed by metastatic patients. Results from serum and urine experiments showed increased levels of **miR-107** in non-metastatic patients compared to HD.	[117]
Prostate	Serum (Exosomes)	Training set 1: PCa (n = 14) mPCa (n = 6) BPH (n = 20) HD (n = 20) Training set 2: PCa (n = 31) mPCa (n = 20) HD (n = 40)	Diagnosis	Serum exosomal **miR-141** was significantly higher in PCa patients compared withBPH and HD. In addition, expression levels were significantly higher in mPCa compared with localized PCa.	[118]
Prostate	Serum (Exosomes)	Discovery set: aggressive PCa (n = 6)BPH (n = 3) HD (n = 3) Training 1: PCa (n = 44) BPH (n = 4) HD (n = 8) Training 2: PCa (n = 25) BPH (n = 21) Validation 2: CRPC (n = 43)	Diagnosis Prognosis	**miR-1246** expression was a significant parameter to discriminate between HD and PCa cases with 100% specificity and 75% sensitivity Patients with aggressive PCa or positive lymph node metastasis showed higher expression of **miR-1246** than normal PCa and non-metastatic patients, respectively.	[119]
Prostate	Plasma (Exosomes)	Discovery set: CPRC (n = 23) Validation set: CPRC (n = 100)	Prognosis	Higher levels of **miR-1290** and **miR-375** were significantly associated with poor OS.	[120]
Prostate	Urine (Exosomes and non-exosomal EVs)	PCa (n = 60) HD (n = 10)	Diagnosis Prognosis	Significant differences were found comparing results in HD and low risk PCa group with intermediate and high-risk PCa groups for **miR-21, miR-375, and let-7 c.** Levels of **let-7c** were significantly associated with clinical stage (T1c vs. T2/T3) and **miR-141** with Gleason score.	[89]
Prostate	Urine (Exosomes)	PCa (n = 14) HD (n = 20)	Diagnosis	Quantification of **miR-19b** in urine exosomes can be used as a primary or at least an auxiliary criterion for the diagnosis of PCa with 95% specificity, 79% sensitivity.	[121]
Prostate	Urine (Exosomes)	PCa (n = 35) HD (n = 35)	Diagnosis	The analysis of **miR-574**, **miR-141**, and **miR-21** in urinary exosomes revealed significant upregulation in PCa patients compared with HD.	[122]

Abbreviations: BPH (Benign Prostate Hyperplasia), CRPC (Castration Resistant Prostate Cancer), EVs (Extracellular Vesicles); HD (Healthy Donors); mPCa (metastatic Prostate Cancer), OS (Overall Survival); PCa (Prostate Cancer), RP (radical prostatectomy).

miRNAs. Statistical packages analyze miRNAs sets with a constant expression in all samples; and all those will be used as housekeepings. This factor is calculated by a geometric average of multiple internal control miRNAs, as it has been described for mRNA by Dr. Vandesompele et al [134].

The number of sequences (reads) should be high for two main reasons. (i) The ligation of adapters bias during sequencing libraries preparation; underestimate the abundance of the most known miRNAs due to an inefficient miRNAs ligation with sequencing adapters [152]. (ii) miRNAs comprise one of the most abundant classes of Small RNAs [153] and with a specific size selection (130–170 bp; and without adaptors and 16–56 bp) the proportion of reads could be higher, but will not exceed a 30–40% of the total sequence. For these reasons; MiSeq system, Nextseq 550 system (Illumina, CA, USA) or Ion S5™

Table 3
Microarray for detection of miRNAs and their characteristics.

Microarray	Company	Characteristics
Human miRNA Microarray. V3	Agilent	4449 miRNAs based on miRBASE public database. Release 9.
GeneChip™ miRNA 4.1	Affymetrix (Thermofisher)	2578 human miRNAs and 1996 human snoRNAs and scaRNA (“small Cajal body-specific RNA”)
Human miRNA Oligo chip10 v21	Toray	2565 human miRNAs
TaqMan™ Array Human MicroRNA A + B Cards	Thermofisher	874 human miRNAs
miScript miRNA PCR Arrays	QIAGEN	Pathway or disease focused panels of miRNA assays (< 96 or < 384)
miRStar™ Cancer Focus miRNA PCR Array (H)	Arraystar Inc	184 human microRNAs
miRCURY™ LNA™ Universal RT microRNA PCR	Exiqon (QIAGEN)	No longer available
MicroRNA Expression Profiling V2	Illumina	No longer available

Footnote Table 3. All details from this table are selected in the manufacturer protocol of each commercial brand.
Abbreviations: MO (non-metastasis); M1 (metastasis).

(Thermo Fisher Scientific, MA, USA) are the ideal equipments for working with miRNAs [154–156].

We are confident that once the potential markers of PCa will be found through NGS studies, as well as, extended in other pathologies, we will be able to study predictive, prognostic and diagnostic markers using dPCR [135,136]. This will allow the detection of miRNAs by dPCR in daily clinical practice, just using a simple blood sample test. There is proven evidence in the study of urine samples from 11 PCa patients, where RNA was extracted and total RNA was sequenced. In this previous study, the lethal-7 family (involved in cell division and differentiation) decreased its expression. Dr. Guelfi et al. described several miRNAs for PCa detection (let-7b-5p, let-7c-5p, let- 7d-5p, let-7e-5p, let-7f-5p, let-7g-5p and let-7i-5p). This study confirmed the use of miRNAs as a non-invasive biomarker in PCa diagnosis, which could reduce the invasiveness of the actual clinical strategy [137].

Several pre-analytical and analytical issues are needed to be taken into account for having a homogenous analysis and data interpretation. For improving this pitfall we could enumerate some proposals: i) Homogeneous and stable collection and storing of the samples; mainly avoiding hemolysis; ii) Clotting time should be the same for all the processed samples, because it affects minimal miRNAs levels; iii) Temperature storage conditions [4 °C or- 80 °C] should be taken into account by its great effect in miRNAs levels; iv)The use of the anticoagulant in blood samples; v) The type of sample blood or serum, there are described differences between miRNA concentration and composition in these both samples [138,139]; vi) extraction methods, profiling platforms, and analysis should be standard to avoid false reports [140]. That is why some collection and extraction protocols are suggested for this purpose such as: i) PAXgene™ blood RNA tubes and Tempus blood RNA tubes for the collection; ii) Chemical extraction method based on concentrated chao-tropic salts such as guanidine thiocyanate (e.g. Trizol and QIAzol® reagents) that lyse cells and inhibit RNAse; are the ones suggested for improving the efficiency; iii) NGS detection technologies have mainly focused on Sequencing-by-synthesis" (Illumina CA, USA) which is widely used for the discovery of novel miRNA signatures mainly by the depth of sequence data obtained. However, post-analytical processes such as miRNAs expression data such as the bioinformatics analysis and normalization strategies are also reporting variable [139].

Studies performed by Jacopo Marzi et al. [138] proposed a test named miR-Test for standardizing circulating miRNAs levels using 13-miRNA and 6-HK models and taking into account factors to avoid loss by hemolysis or wrong storage or collection conditions. Finally, suggesting that for circulating miRNA is heavily influenced by all previous preanalytical issues and that is crucial if these molecules would be implemented in clinical practice [138]. Previous data published by Witwer indicated controversial roles reported in miRNA-141 indicating that a careful optimization and standardization of preanalytical and analytical methods is needed to ensure reliable data [140].

As a summary of the impact and technologies for miRNAs we have included a Supplementary Fig. 1 with these details.

5. Conclusions

In PCa, most of the conclusive biomarkers are still being invasive with procedures involving pain and other controversial clinical adverse effects. Nowadays we are closer for approaching to precision medicine and to it is routinely use in clinical practice. This disease treatment and prevention approach takes into account each person´s variability; including genomic data of each single patient. Moreover, another objective of current medicine is providing effective and non-invasive methodologies for screening, monitoring or following-up a disease, creating optimal biomarkers.

Several miRNAs have been reported to be deregulated in body fluids of PCa patients compared to healthy controls (as miR-375), as well as altered in different stages (as miR-141) of PCa or treatments; all these previous data suggest that miRNAs can be a promise non-invasive biomarker in PCa [141]. However, the existence of an efficient biomarker in some urological tumors such as PCa is still a challenge; and it can be proved by the previous controversial data along the text. Many studies have shown novelties in outperforming PSA as the combination of miR-100 and miR-200, but they are not used yet in clinical practice. It is known there are many efforts in searching new promising biomarkers for reducing invasive and unnecessary biopsies in PCa and miRNAs could benefit these non-invasive strategies.

Here we reported the current status methodologies, strategies and controversial points, of using free miRNAs (in blood and urine mainly) and exomiRNAs as novel non-invasive biomarkers. Non-invasive strategies are useful for current guiding therapies disease risks, predicting clinical outcomes and the benefits of the use of these molecules in the improvement of current biomarkers.

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Conflicts of interest

The authors declare that they have no conflicts of interest with the contents.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mrrev.2019.05.005.

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