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Review Article

microRNA biomarkers in body fluids of prostate cancer patients

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ABSTRACT

The abundance of miRNAs – small non-coding RNAs involved in posttranscriptional regulation of gene expression – in tissues and body fluids of cancer patients hold great promise to identify specific biomarkers, which may be useful for early diagnosis as well as to predict the clinical outcome and treatment response. For the extraction and quantification of miRNAs from cells and tissues, present technologies for transcriptome analyses like microarrays, quantitative real-time PCR or next generation sequencing can be applied. However, the analyses of miRNAs in body fluids like serum or urine is still a challenge with respect to the nucleic acid recovery from very limited sources of biomaterial, normalization strategies and validation using independent technologies. The presence of specific miRNA patterns in body fluids like serum of cancer patients suggests a promising role of these molecules as surrogate markers. However, the majority of miRNA studies were addressed in relatively small patient cohorts limiting the validity and the clinical application of potential miRNA biomarkers or signatures. We reflect the critical steps to translate miRNA biomarker into clinical routine diagnostics and present future aspects for the fast, robust and standardized quantification of miRNAs in body fluids.

1. Introduction

The discovery of miRNAs is an emerging field in cancer research with potential novel applications in diagnostics and therapy [1]. Specific miRNAs aberrantly expressed in tumor tissues play an important role in cancer onset and disease progression by targeting cancer-relevant coding genes. The intratumoral increase of specific miRNAs can be reflected in body fluids of the patients. Moreover, circulating miRNA pattern in patients may be associated with the genetic predisposition or immune system escape. Therefore, miRNAs involved in early tumorigenesis, tumor progression and metastasis represent promising diagnostic or prognostic marker, respectively [2]. Current methods for prostate cancer detection, like PSA screening, lead to significant overtreatment [3]. Thus, the requirements for novel biomarkers should address the stratification of patients with severe tumor diseases which become clinically relevant and need therapeutic intervention. Several gene and miRNA biomarkers and signatures in tissues were described to be associated with prostate cancer onset or progression. Despite the increasing number of miRNA reports, the analysis of circulating miRNAs is still in its infancy. Previous comprehensive miRNA biomarker screening in serum and plasma obtained promising results, but missing standards for sample preparation, quality control and normalization may hamper robustness of measurements and accuracy of putative disease biomarkers. Therefore, further insights into miRNA abundance and characteristics in surrogates are necessary to overcome limitations of the present detection methods.

2. Current status of biomarkers for prostate cancer

2.1. Diagnosis of prostate cancer

Prostate cancer is a common tumor disease in western countries and a leading cause of cancer-driven mortality in men. This entity often comprises slowly growing tumors, which are diagnosed at the age of 70 or even later. At this age, the incidence of prostate cancer is very high (30–70%), but the majority of prostate cancer cases behave clinically insignificant [4]. Of note, overdiagnosis and overtreatment are the key challenges for prostate cancer patient care. First, no malignancy was found in biopsies for the majority of men with increased blood PSA level >4 ng/ml, which indicated a high rate of unneeded interventions. Second, current treatment strategies like radical prostatectomy and radiation therapy still

Abbreviations: BPH, benign prostatic hyperplasia; FFPE, formalin fixed paraffin embedded; FISH, fluorescence in situ hybridization; miRNA, microRNA; PCA3, prostate cancer gene 3; PSA, prostate specific antigen; qPCR, quantitative real-time PCR.

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have the risk for erectile dysfunction and urinary incontinence. Two large clinical trials reported conflicting results of PSA screening on prostate cancer mortality [5,6]. In October 2011, 40 years after PSA was identified, the U.S. Preventive Services Task Force (USPSTF) recommended against PSA screening for prostate cancer detection [3]. So far, no prognostic biomarker entered routine diagnostics of prostate cancer. The prospective requirements for prostate cancer biomarkers address the stratification of patients with aggressive tumors which need therapeutic intervention. For this purpose, robust gene and miRNA biomarker associated with clinical significant prostate cancer may help to improve patient stratification or may serve as diagnostic adjunct in combination with histopathological and clinical parameters (Fig. 1.).

2.2. Genetic alterations in prostate cancer: objectives for diagnostic and prognostic biomarkers

The tumorigenesis and progression of prostate cancer is accompanied by distinct molecular alterations. For example, early chromosomal rearrangement leads to the *TMPRSS2-ERG* fusion gene in about half of the patients, and can be detected by FISH or ERG immunohistochemistry [7–9]. Such cancer-specific fusion genes represent promising targets for molecular diagnosis and targeted therapy approaches [10]. Further common genetic alterations concern epigenetic silencing like the *GSTP1* promotor hypermethylation or the loss of tumor suppressor genes *p27* and *NKX3.1* in localized prostate cancer [11,12]. *PTEN* loss is a frequent event towards a metastatic disease, especially in combination with *ERG* dysregulation [13]. In late disease stage, aberrant androgen receptor (AR) signaling, caused by *AR* mutation, amplification or aberrant splice variants, is a prerequisite for castration resistant prostate cancer [14].

Common genetic alterations in tumors have also an impact on the activity of specific miRNAs. Genome-wide epigenetic silencing in prostate cancer was suggested to affect miRNAs in 30% of the identified loci [15]. For example, aberrant methylation leads to a downregulation of miR-145 [16]. miR-16 was also found to be downregulated in prostate cancer compared to benign tissues or benign prostatic hyperplasia (BPH) [17–19], and was controversially discussed as putative diagnostic marker in surrogates

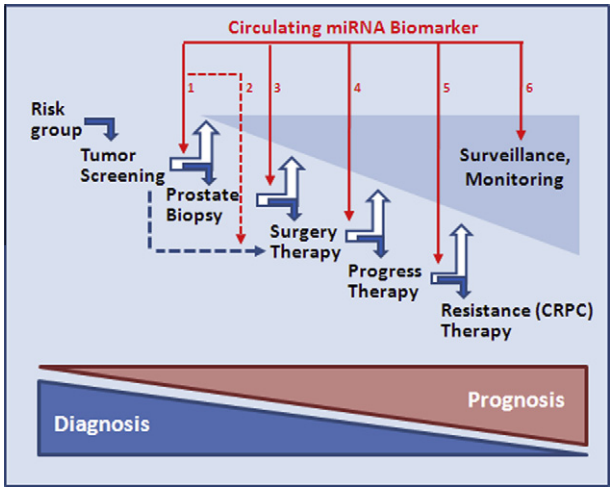

Fig. 1. Circulating miRNA biomarker will be particularly useful to assess the disease prognosis in order to select patients with a high-risk for clinically significant prostate cancer. Those biomarkers might be beneficial in different scenarios: prevention of unneeded biopsies (1,2), selection of high-risk patients for therapeutic intervention (3), detection of metastatic recurrence (4), prediction of therapy resistance (5), and active surveillance and monitoring the disease onset and progress (6).

[20–22]. Thus, miRNA alterations based on amplification, deletion or epigenetic silencing may lead to the development of clinical useful diagnostic or prognostic biomarkers.

2.3. Unraveling gene and miRNA expression signatures in prostate cancer

In clinical practice, preoperative PSA, tumor stage, Gleason grade and surgical margins are used as prognostic factors and are essential to select the best treatment strategy. After surgery, biochemical (PSA) relapse indicates further disease progression and dissemination. These parameters have been used to screen for prognostically relevant gene and miRNA signatures upon expression profiling. In contrast to well characterized tumor entities like breast cancer, expression profiling of prostate tumors did not unravel robust molecular subtypes. However, several tumor tissue derived signatures based on microarrays have been associated with prognostic parameters like Gleason score, biochemical recurrence and lethality [23,24]. Moreover, integrative gene and miRNA analyses may improve tumor characterization and patient stratification [25–28]. Such promising tumor-related gene and miRNA expression signatures should be validated in larger clinical trials and, in best case scenario, be applied to less-invasive diagnostic techniques.

Large scale miRNA expression analyses using microarrays indicated a common deregulation of miRNAs in tumors compared to their benign counterparts [29,30]. The miRNA profiles fairly reflect both the origin of tissues and the malignant phenotype. In prostate cancer, several studies showed differences in the abundance of miRNAs in localized or metastatic prostate cancer compared to benign prostate epithelium or BPH, [17–19,25,27,30–34]. More than 180 different miRNAs were described to be deregulated in prostate cancer in at least one screening study. More than 100 miRNAs appeared only in single studies. Of note, the miRNA coverage of different platforms used in these studies strongly varies (between 114 and 723 miRNAs), and the number of finally measurable targets in individual platforms is often unclear. In later disease stage, miRNA signatures were identified to be associated with a metastatic or hormone-refractory disease [17,31]. For example, Martens-Uzunova et al. described a 25 miRNA signature which correctly classified between a local and metastatic disease in about 80% of the patients. Several miRNAs were also shown to be deregulated and functionally relevant across different cancer diseases [35]. For example, miR-21 upregulated in different solid tumors like prostate cancer was shown to act as oncogene by targeting tumor suppressors like *PTEN* and *PDCD4*. miRNA-21 activation might enhance general processes of tumor invasion and metastasis by promoting extracellular remodeling in the tumor environment [36]. In contrast, miR-15a and miR-16-1 are transcribed as a cluster, which is frequently downregulated in prostate cancer [37]. These miRNAs control transformation and cell proliferation by targeting oncogenes like *BCL-2* and *CCND1*.

For most of the deregulated miRNAs it is still unclear whether they can serve as diagnostic or prognostic markers. However, aberrantly expressed miRNAs in tumor tissues may be reflected in patient body fluids, and therefore represent a promising source for putative surrogate marker.

3. miRNAs as non-invasive tumor marker in body fluids

3.1. miRNome in human body fluids

miRNAs (miRNAs) were discovered in the early 2000s as a new class of small functional RNA molecules, which play an important role in posttranscriptional gene regulation. Transcribed microRNA (miRNA) sequences and their reverse-complement base pairs form

double stranded RNA hairpin loops, defined as precursor miRNAs, which are exported from the nucleus and processed to about 22 nucleotide long mature miRNAs. In the miRBase database, Release 18 (Nov 2011), 1527 different hairpin precursor miRNAs are assigned in the human genome [38].

Detection of miRNAs in body fluids represents a promising non-invasive diagnostic utility for cancer diseases. Between 200 and 500 miRNAs were detected by qPCR in different human body fluids like plasma, urine or breast milk [39]. The utility of distinct body fluids for miRNA biomarker discovery may be dependent on the disease origin. For example, miRNAs in sputum, bronchial lavage or exhaled breath may be useful to reflect early stage lung cancer, whereas miRNAs in the urine have been explored for biomarker associated with urological cancer. Circulating miRNAs were firstly described in serum and plasma as promising biomarkers for cancer diagnostics [22,40,41]. Interestingly, circulating miRNAs are robust against RNase in cell-free compartments like serum and resist high variability of pH and temperature [22]. The release of miRNAs into the blood stream and their functional consequences are poorly understood. It has been shown that mRNA and miRNA can be integrated into microvesicles and or associated with protein complexes [42,43]. In cancer patients, distinct miRNA composition in microvesicles may drive invasion, angiogenesis and metastasis [44,45].

So far, peripheral blood has been mainly used to analyze cell-free miRNAs in cancer patients [46]. A good concordance of miRNA expression was shown between serum and plasma of the same individuals [22]. Here, the miRNA abundance was not affected by residual blood cells or cell debris. Similarly, it has been shown that the miRNA profiles differ between serum and blood cells [40]. However, the variability of miRNA abundance in blood components like serum, plasma or blood cells is controversially discussed. miRNA profiles from blood cells may be confounded by the heterogeneous composition of hematopoietic cells and their specific miRNA profiles [47]. Furthermore, it was shown that red blood cells and the degree of hemolysis influence the plasma miRNA content [48,49]. Potential miRNA biomarkers were also identified in circulating tumor cells previously sorted from whole blood [50]. Thus, standardized protocols for the collection, processing, shipment and storage of body fluids are prerequisites to ensure the reliable measurement of miRNAs.

3.2. miRNA detection: prospects and challenges

The extraction and quantification of circulating miRNAs is challenging with respect to the low recovery, standards for quality control, normalization and statistical analysis (Fig. 2). For example, the low concentration of cell-free circulating miRNAs from 100 µl serum or plasma cannot be accurately calculated by spectrophotometry [51]. Therefore, RNA extraction protocols usually recommend a constant starting volume of the body fluid. A frequently adopted protocol describes the use of Trizol and Chloroform for denaturation and separation of proteins followed by miRNA purification from aqueous phase by commercially available column-based extraction kits [51]. Further protocols are established for the miRNA detection in peripheral blood cells [52] or in exosomes isolated from body fluids [53]. Synthetic non-human miRNAs as spike-in control were recommended to monitor the extraction efficiency and to normalize sample-to-sample variations [22,54]. However, spiked and endogenous miRNAs may behave different with respect to the recovery rate or the subsequent efficiency and dynamic range of quantification techniques. Alternatively, human miRNAs like RNU6B or miR-16 were often used as endogenous controls to account for technical variances and biological variability. But, single miRNAs might respond to different physiological and pathological conditions. For example, serum miR-16 level was found to be associated with surgical margin positivity in prostate cancer patients [21]. Comprehensive circulating miRNA profiling studies in distinct disease areas are rare and often miss robust standards for miRNA preparation and quantification. Bianchi and colleagues performed a large screening study in serum of 174 patients using qPCR low-density miRNA plates [55]. Here, miRNAs were quality assessed to account for sequence verification, robust and reproducible detection (sensitivity), PCR efficiency (linearity) and potential preoperative bias based on the patient cohorts. Furthermore, different normalization procedures revealed a good concordance between median normalization and housekeeping method (miR-197, miR-19b, miR-24, miR-146, miR-15b, miR-19a).qPCR technology is mostly used for screening and validation of putative miRNA biomarker in surrogates, which is a drawback for the objective evaluation of technical limitations. So far, only few studies reported the use of next generation sequencing technology, which may be due to the lower sensitivity. Solexa sequencing enabled the detection of about 100 serum miRNAs [40,56]. Alternative miRNA quantification technologies have been recently described like the use of fluorescence-labeled beads [57], modified nanoparticles [58] or electrochemical genosensor without bias-prone PCR and labeling reaction [59]. The latter method is based on the hybrid formation between the circulating microRNA and its inosine substitute capture probe on an electrode surface. The subsequent guanine oxidation generates an electrical signal that

Experimental Workflow	Sources and Methods	Critical Issues	Recommendations
1. Biomaterial	serum, plasma, blood, urine, saliva	withdrawal, storage, hemolysis, cell heterogeneity	standard operating procedures, cell- or body fluid specific markers
2. miRNA extraction	phenol/chloroform and column-based purification kits	efficiency, reproducibility, high-throughput	spike-in controls, column affinity, small RNA recovery test
3. miRNA enrichment	preamplification	amplification bias	concordance: miRNA abundance, non-amplified vs. amplified
4. miRNome profiling	microarray qPCR sequencing (NGS)	sensitivity, cross hybridization, PCR specificity and efficiency, adaptor dimers, multiplexing	spike-in analysis, hybridization controls, PCR efficiency test, independent validation
5. Data processing	normalization background correction NGS: annotation	normalization method, sequence annotation	quantile or median normalization, valid small RNA internal controls, NGS: valid mapping algorithms
6. Data analysis	differential expression analysis, classification, survival analysis, NGS: novel small RNA screen	multiple testing, preselected cohorts, validation	testing correction, complete follow-up data, independent validation of miRNAs / signatures

Fig. 2. Experimental workflow, methods, critical issues and recommendations for circulating miRNA quantification in body fluids.

is evaluated by a differential pulse voltammeter. The future application of circulating miRNAs into clinical routine diagnostics will depend on the efforts to simplify and standardize the methods for sample preparation, detection and data processing.

3.3. Circulating miRNAs in prostate cancer patients

Few comprehensive miRNA profiling studies have been focused on circulating miRNA biomarkers in prostate cancer patients (Table 1). Several studies reported comprehensive miRNA screening using qPCR or microarray technology [54,60–63] or performed quantification of single miRNAs in distinct prostate cancer patient cohort and healthy individuals [21,22,64–66]. miRNAs were mostly studied in serum and plasma of men. Bryant and colleagues screened for diagnostic and prognostic miRNAs in plasma samples, and were also able to identify two of five miRNAs as putative diagnostic markers in urine samples [60]. The overlap of miRNA candidates between different studies is very small. Here, it is likely that different miRNA platforms, quantification protocols, statistical cutoffs, patient preselection and small cohort size limits this comparison.

Of note, two promising miRNAs, miR-141 and miR-375, were suggested as diagnostic and prognostic marker across independent studies. Increased plasma miR-141 level was firstly described in patients with metastatic prostate cancer compared to healthy individuals [22]. Additionally, circulating miR-141 was moderately correlated with serum PSA level. Of note, elevated serum level of miR-141 have been independently confirmed for metastatic prostate cancer disease, and, together with miR-375, associated with further prognostic parameters like higher Gleason score and positive lymph node status [54]. The higher abundance of circulating miR-141 in metastatic compared to local prostate cancer disease was again confirmed using patient plasma [65]. Recently, miR-375 and miR-141 have been identified in serum of prostate cancer mouse models and further analyzed in cancer patients [63]. Here, both miRNAs were found to be elevated in serum of men with metastatic castration-resistant prostate cancer. In summary, four independent studies analyzed circulating miRNAs in about 240 prostate cancer patients and 70 healthy controls and pointed to either miR-141 and miR-375 or both as diagnostic and prognostic marker. Furthermore, intratumoral expression of miR-375 and miR-141 was increased compared to benign tissues [18,25,27,31,33,34,54] and associated with biochemical relapse [63]. The function of both miRNAs in prostate cancer cells is poorly understood. miR-375 increase was described to inhibit Sec23A protein, and stimulated cell proliferation in prostate cancer cell lines [67]. miR-141 was found to be upregulated upon androgen stimulation and its activation enhanced growth of LNCaP cells [68]. Therefore, both miRNAs may be aberrantly activated in prostate cancer and functionally involved in disease progression. It has been shown that miR-141 and miR-375 increase during tumor progression is reflected in the blood of the patients. The detection of such miRNAs in patient's surrogate represents a promising approach or diagnostic adjunct to detect severe prostate cancer disease.

Further circulating miRNAs have been associated with prostate cancer like miR-21 and miR-221. Deregulation of these miRNAs were described in tumor tissue [17–19,25,27,30,31,33,34] and serum [64–66] of prostate cancer patients. The identification of downregulated miRNAs in patient's surrogate may be more challenging with respect to the sensitivity and valid detection standards. Further commonly increased miRNAs in prostate tumor tissues like miR-20a, miR-25, miR-93 and miR-106b represent good candidates for their detection in body fluids. For example, miR-93 was found to be elevated in sera of high-risk prostate cancer patients [62]. However, larger validation studies are needed to verify putative miRNA biomarkers for their association with a certain disease status. Furthermore, blood-based biomarker may be influenced by other physiological or pathological conditions like viral infections. For example, abundance of miR-375, at present one of the most

Table 1
Circulating miRNA quantification studies in body fluids of prostate cancer patients and controls.

References	Clinical parameter	Body fluid	Cohort design	miRNA quantification platform	Normalization	miRNA candidates
Brase et al. (2010)	Prognosis: low vs. high grade	Serum	Screening $n = 21$, validation $n = 116$	qPCR plates (667 miRNAs), qPCR assays	Spike-in cel-miR-39, -54, -239	miR-141, -375
Moltzahn et al. (2011)	Diagnosis tumor vs. healthy	Serum	Screening $n = 48$, validation same cohort	qPCR plates (677 miRNAs)	Median	miR-24, -26b, -30c, -93, -106a, -223, -451, -874, -1207–5p, -1274a
Selth et al. (2011)	Diagnosis, prognosis tumor vs. healthy, low vs. high grade	Serum	Screening murine TRAMP model , validation $n = 50$	Microarrays (609 murine miRNAs), qPCR assays	cel-miR-39	Diagnosis miR-141–298, -346, -375, prognosis miR-141, -375
Bryant et al. (2012)	Diagnosis, prognosis tumor vs. healthy, local vs. metastatic	Plasma micro-vesicles, urine	Screening $n = 106$, validation $n = 119$, urine: $n = 135$	qPCR low-density plates (742 miRNAs), qPCR assays	Screening: inter-plate calibrators, validation: cel-miR-39, urine RNU44/48	Diagnosis miR-107, -574–3p (plasma, urine), prognosis miR-141, -375 (plasma)
Chen et al. (2012)	Diagnosis tumor vs. BPH, healthy	Plasma	Screening $n = 42$, validation $n = 178$	Microarrays (1146 miRNAs), qPCR assays	Screening quantile validation RNU6B	let-7e, -7c, miR-30c -622, -1285
Mitchell et al. (2008) #	Diagnosis tumor vs. healthy	Plasma	No screening, $n = 50$	qPCR assays miR-100, -125b, -141, -143, -205, -296	Spike-in: cel-miR-39, -54, -238	miR-141
Zhang et al. (2010)	Prognosis local vs. metastatic	Serum	No screening, $n = 56$	qPCR assay miR-21	RNU6B	miR-21
Agaoglu et al. (2011)	Diagnosis, prognosis tumor vs. healthy, local vs. metastatic	Plasma	No screening, $n = 71$	qPCR assays miR-21, miR-141, miR-221	RNU1A	Diagnosis miR-21, -221, prognosis miR-141
Mahn et al. (2011)	Diagnosis tumor vs. BPH, healthy	Serum	No screening, $n = 83$	qPCR assays miR-26a, -32, -195, let7i	cel-miR-39	miR-26a
Shen et al. (2012)	Prognosis low vs. high grade	Plasma	No screening, $n = 82$	qPCR assays miR-20a, -21, -141, -145, -221	Absolute quantification	miR-20a, -21, -145, -221

promising circulating miRNA biomarker for prostate cancer, is described to be affected by HPV infection [69]. Therefore, discriminative power of single miRNAs may be restricted by those confounding factors. A multi-marker miRNA model or robust miRNA signature for the stratification of specific diseases may overcome such limitations.

4. Conclusions

The identification of circulating miRNA biomarkers in body fluids is far from being exhausted. Specific disease-associated miRNA patterns have been identified in affected tissues and cells, but also in body fluids of cancer patients. In prostate cancer, most of the circulating miRNA studies were done using serum or plasma of the patients. Here, promising results were obtained for potentially prognostic miRNA biomarkers (e.g. miR-141 and miR-375), which have been associated with aggressive tumors and metastatic disease in independent studies. Such findings have to be validated in larger patient and control collectives, e.g. to account for individual confounding factors like prostatitis, BPH, other common age-dependent diseases and epidemiological characteristics. Furthermore, urine-based test for the prostate cancer gene PCA3 has already been used to detect prostate cancer. Specific miRNA patterns in the urine may also reflect early or advanced prostate cancer disease, but no comprehensive study for circulating miRNA in urine was reported so far.

In summary, circulating miRNAs may represent promising biomarkers for early detection of a cancer disease or to predict clinical outcome. However, the quantification of circulating miRNAs in prostate cancer is in its infancy. The dozen studies are very heterogeneous with respect to the experimental design, patient cohort and clinical question, and therefore lack comparable results. A major weakness of present retrospective biomarker studies is the lack of comprehensive and meaningful follow up data of the patients. Only very few biomarker studies focused on prognostic endpoints like cancer-related death or progression free survival, but instead used surrogates like PSA biochemical relapse. The future application of circulating miRNA biomarker into routine clinical practice will depend on the establishment of robust methods and standards for miRNA detection, as well as the validation of promising candidates in large prospective studies.

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