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Circulating nucleic acids as biomarkers of prostate cancer

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Abstract

Prostate cancer, the most common cancer of western men, requires new biomarkers, especially given that the benefits of PSA testing remain uncertain. Nucleic acids can now be accurately and sensitively detected in human blood. Over the last decade, investigations into utility of circulating cell-free miRNA, DNA and mRNA as novel biomarkers have expanded exponentially. In the near future, they may be routinely used to accurately diagnose cancers, stratify indolent from aggressive disease and inform treatment decisions. However, advancement of such tests into clinical settings is hampered by technical problems with assay specificity and sensitivity, and small study sizes. This review highlights the different forms of circulating nucleic acids and those that show the most potential as viable biomarkers for prostate cancer

Keywords

biomarkers; cell-free DNA; circulating nucleic acids; microRNA; miRNA; mRNA; prostate cancer

The need for new biomarkers for prostate cancer

Prostate cancer is the most commonly diagnosed male cancer in the western world, with over 40,000 cases in the UK diagnosed each year [101]. It largely affects older men, with a median age at diagnosis of 72 years. While many men are diagnosed with the disease far fewer will actually die of it. For men in the USA, overall lifetime risk of developing prostate cancer is one in six; however, the mortality risk is markedly lower at one in 36 [1]. This creates a difficult paradigm whereby many patients are diagnosed with prostate cancer and many will have indolent disease. Without markers to predict which tumors will become

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aggressive, many patients will receive therapies, which have multiple deleterious side effects and for which little or no therapeutic benefit will be gained.

In the last two decades prostate cancer mortality has decreased, in part due to the introduction of PSA testing, which has forever changed the way that prostate cancer is managed. PSA detection in serum is currently the gold-standard biomarker for diagnosis and response to treatment. Currently, PSA combined with digital rectal examination is used to indicate a possible diagnosis of prostate cancer, warranting further investigation with transrectal biopsy. However, there are some well-known limitations to the use of PSA as a biomarker. Elevated serum PSA levels are not specific to prostate cancer and are frequently present in men with other diseases of the prostate, such as benign prostatic hypertrophy (BPH) and prostatitis. This results in a large false-positive rate, with less than 50% of men who have a prostate biopsy following a raised PSA result actually being diagnosed with prostate cancer [2]. As prostate biopsy carries risks, especially of infection, this level of false positives is unacceptably high [2]. In addition, because of its lack of specificity, a very low PSA level does not completely rule out prostate cancer and there is also a false negative rate, of approximately 15%, where a negative PSA reading (0–4 ng/ml) has not indicated the underlying presence of cancer [3]. There have been numerous strategies proposed to improve PSA diagnostic performance, such as free PSA, PSA velocity, and age- or race-specific PSA reference ranges. Although these have been used by clinicians and continue to be investigated there is no consensus on their use as none have yet been shown in clinical trials to decrease unnecessary biopsies or improve outcomes [4]. The cost of overdiagnosis of prostate cancer – both on a personal health level and financially for healthcare systems – has driven a great deal of attention toward finding novel noninvasive markers. There are a number of other diagnostic tests that have potential and are currently being trialled, for example the urinary levels of PCA3 if raised have been shown to correspond to a diagnosis of prostate cancer [5]. There are also ongoing trials looking at the expression of the *TMPRSS2*:*ERG* fusion gene in cells discharged into the urine following a rectal exam, as this fusion gene is rarely found in cells of men without prostate cancer [6]. Prostate cancer management would benefit greatly from new biomarkers for diagnosis, prognosis and treatment response.

Since the first publications about miRNAs relating to prostate cancer in 2007, there have been more than 100 articles on the subject reflecting the hope that miRNAs may be the future for biomarkers of this disease. These have been joined by reports of other circulating nucleic acids, cell-free DNA and mRNA as potential biomarkers. In this review we strive to highlight and summarize the potential role of circulating nucleic acids as novel biomarkers in prostate cancer.

Cell-free DNA as biomarkers

There is currently scant evidence as to the clinical utility of cell-free circulating DNA or mRNA as prognostic, diagnostic and/or predictive biomarkers in prostate cancer. Such markers are attractive to clinicians and scientists alike owing to their potential for minimally invasive detection and monitoring of disease pathogenesis, but currently present considerable technical challenges in terms of sensitivity, specificity and/or nucleic acid

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stability. In contrast to mRNA, circulating DNA-based tumor markers exhibit greater stability and enhanced tumor specificity, potentially enabling tumor grading/staging, prognostic estimation and aiding therapeutic decision-making. The first report of cell-free circulating DNA in blood plasma and serum was in 1948 [7] and it was later demonstrated that cancer patients have higher levels of cell-free DNA than normal controls [8–10]. Indeed, it has recently been established that prostate cancer patients have threefold higher levels of circulating DNA than subjects with BPH [11]. Importantly in the context of biomarker identification, cell-free circulating DNA has been correlated with pathological stage [10], Gleason score, status of surgical margins and extraprostatic invasion [12], and metastasis [12–14]. However, reports vary widely in their estimations of the proportion of circulating DNA in cancer patient blood that is of direct tumor origin, with figures as low as 3% and as high as 93% reported [15]. However, alterations in cell-free circulating DNA have been shown to match mutations present in primary tumors [16], and thus at least part of the elevated DNA content of cancer patient blood derived from the tumor. It is likely that the percentage of blood cell-free DNA that is tumor-derived varies widely and is dependent on type of tumor, stage, metastatic status and treatment regimens, among many other factors. DNA levels in plasma reflect a number of cellular processes, including apoptosis, necrosis and/or active release from circulating tumor cells. What is widely accepted, however, is that, in most cases, the majority of circulating cell-free DNA is derived from healthy cells [15,17].

In prostate cancer, three types of DNA alterations have been investigated as plasma/serum biomarkers. These are mitochondrial DNA (mtDNA) mutations [11,18–20], microsatellite instability (MI) [14,21–24] and gene promoter hypermethylation [10,12,21,24–32]. These represent potentially attractive biomarkers: mtDNA is present at far higher copy number (20–200 copies per cell) than genomic DNA (two copies per cell) and may theoretically be more readily detectable, and methylation-specific PCR (MSP) for detection of gene promoter hypermethylation is a highly sensitive application, requiring only 0.1–0.001% of serum DNA to be of tumor origin [33]. In addition, such markers largely represent tumor-specific events, reducing the risk of false-positive detection.

Hypermethylation events

Hypermethylation of CpG islands within the promoter of the gene encoding GSTP1, a tumor-supressor protein involved in detoxification processes, has been described as one of the earliest events in prostate carcinogenesis and leads to loss of gene expression. This epigenetic event has been investigated as a cell-free DNA-based biomarker for prostate cancer diagnosis [33], an application for which it is theoretically ideally suited, being detectable in up to 100% of prostate tumors [34,35]. Importantly, this event is detected in over 90% of early-stage prostate cancer tumors [33], enhancing its potential as a diagnostic biomarker, or as a complement to PSA testing. Reports are conflicting as to the presence of *GSTP1* promoter hypermethylation (GPH) in BPH and prostatic intraepithelial neoplasia (PIN) patients: in one study, GPH was detected in 30% of benign prostate patients [18], whereas in a second, GPH was entirely absent from benign prostate tissue, but found in two-thirds of high-grade PIN cases [36]. Several other studies support the specificity of GPH as a prostate cancer biomarker and failed to detect GPH events in benign prostate tissue [33–35].

Indeed, Nakayama *et al.* found no evidence of GPH in laser-capture microdissected sections of normal prostate epithelium or BPH, but 69% of PIN sections and 91% of prostate cancer sections were GPH positive [37], possibly highlighting GPH as a tumor development-specific event. In any case, given the minimal levels of GPH in benign tissue, it is unclear whether this would be detectable in body fluids at all. In addition, quantitative differences between GPH levels in BPH and cancerous tissue are in the order of 150-fold [38]. Thus, if this ratio is retained in serum/plasma, careful definition of a suitable cutoff would likely allow discrimination between prostate cancer and BPH and/or PIN in a blood-based test.

Epigenetic events like GPH can be exploited by MSP, allowing rapid, automatable and sensitive biomarker analysis. One of the first studies to employ these technologies for diagnostic purposes in serum found that GPH was absent in all serum samples from 26 BPH patients, but, in a cohort of 33 prostate cancer patients, GPH was detected in 72% of serum samples and 94% of tumor samples [33]. In a second study, Goessl *et al.* identified GPH in the plasma of 56% of men with $T_{2–3}$ prostate cancer and 93% of men with lymph node-positive and/or metastatic T_4 prostate cancer [33,39], suggesting that quantification of GPH levels may be informative as to disease stage, prognosis and outcome. Indeed, in another study, all patients with locally advanced (T_4) or metastatic disease demonstrated serum/plasma GPH positivity [33], and the specificity of MSP was found to be 100%. Furthermore, GPH was the strongest predictor of PSA recurrence following radical prostatectomy [40], and was correlated with Gleason score and degree of metastatic spread in patients with hormone-refractory disease [29]. Interestingly, GPH analysis performed more sensitively in serum samples than in other body fluids in detecting prostate cancer (50% of ejaculates and 36% of urine samples from prostate cancer patients were positive for GPH), although the numbers in each group were very small. As GPH is a tumor-specific event, and is a feature of cell-free DNA of tumor origin in prostate cancer patients, it may be a valuable biomarker for prostate cancer diagnosis. Also, as prostate cancer is detected by repeat biopsy in up to 30% of men who were not given a diagnosis of prostate cancer upon initial biopsy [41,42], highly sensitive and specific MSP detection of GPH in circulating DNA may identify men with prostate cancer who may otherwise have been missed.

Hypermethylation of two further tumor suppressor genes, *RASSF1A* and *RARB2*, which are hypermethylated in primary prostate tumors [43–46], are correlated with Gleason score and serum PSA, and so have also been proposed as adverse prognostic biomarkers in prostate cancer plasma/serum DNA [24].

In a high-throughput approach, Cortese *et al.* analyzed the circulating DNA methylomes of 19 PC patients, 20 BPH patients and 20 control men by DNA modification-sensitive restriction digestion followed by analysis on micro-arrays containing over 12,000 GC-rich clones. This identified 39 disease-associated changes to circulating DNA modifications, of which seven were validated in an independent patient cohort. Of particular interest was a DNA modification upstream of the *RNF219* gene, which distinguished prostate cancer from benign disease with sensitivity of 61% and specificity of 71% [47]. Furthermore, patients with stage III prostate cancer exhibit significant loss of repetitive pericentromeric DNA on chromosome 10 compared with stage II and control samples, suggesting that chromosome 10 may undergo copy number loss with repetitive DNA elements in advanced prostate

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cancer [47]. In addition, the authors exploited machine-learning applications to develop a multilocus PC biomarker panel, although this was only 72% accurate in distinguishing prostate cancer from benign controls [47].

Trimethylation of histone H3 at lysine 27 (H3K27me3) by EZH2 is an important epigenetic event associated with transcriptional silencing. This event is associated with poor prognosis in many cancers [48], and a recent report provides evidence that global loss or gain of H3K27me3 in prostate tumors may represent an epigenetic biomarker for the disease [49]. For this reason, Deligezer *et al.* assayed levels of H3K27me3 in blood plasma of patients with local, locally advanced and metastatic prostate cancer by quantitative real-time PCR (qPCR) and ELISA [50]. H3K27me3 levels were found to be significantly lower in patients with metastatic prostate cancer than those with local or locally advanced disease [50]. In addition, plasma H3K27me3 levels were able to correctly discriminate metastatic prostate cancer from localized or locally advanced prostate cancer in 74% of cases [50]. These data suggest that plasma H3K27me3 levels may be used in conjunction with PSA levels post-treatment to assess disease progression and/or treatment response in a combination prognostic biomarker approach.

MI in serum/plasma

A number of studies have demonstrated MI in cell-free DNA in the serum or plasma of prostate cancer patients [14,21,23,24], although allelic imbalances in cell-free DNA were also observed in the plasma/serum of BPH patients [21,23]. In 2009, Sunami *et al.* used a panel of six MI markers to assess allelic imbalances in 83 prostate cancer patients and 40 controls, which provided a specificity of 100% in prostate cancer detection, but a much lower sensitivity, detecting one or more of six loss of heterozygosity markers in the serum of only 47% of prostate cancer cases [24]. Similarly, Schwarzenbach *et al.* found that 14 MI markers identified only 45% of 69 prostate cancer serum samples, albeit with a specificity of 100%. However, in a larger study of 230 patients and 43 controls, the same authors used a panel of thirteen MI markers to identify 57% of prostate cancer cases, but at a reduced specificity of 70% [14]. Additionally, increased frequency of MI markers was identified in patients with metastatic prostate cancer [14]. Interestingly, Sunami *et al.* found that a combination of MI markers and promoter methylation assays was more informative and predictive than either marker alone [24]. Indeed, at least one marker of allelic imbalance and promoter hypermethylation was identified in the circulating DNA of 63% of patients, compared with 16% for loss of heterozygosity alone and 34% for promoter hypermethylation alone. The authors of this study propose that such a combined panel of DNA markers may complement serum PSA analysis for prostate cancer diagnosis or monitoring. In fact, while PSA detection alone detected 71% of cases, 89% of prostate cancer cases were detected using a combination of PSA, MI analysis and promoter hypermethylation qPCR-based analysis of circulating DNA without an increased false-positive rate [24]. Introduction of additional markers of MI or gene methylation may be required to increase sensitivity of prostate cancer detection and overcome the high degree of tumor heterogeneity that is often observed in prostate cancer, and to accommodate differences in clearance rates of circulating tumor-associated DNA.

mtDNA in plasma/serum

Studies have identified similar levels of mtDNA in the serum or plasma of subjects with BPH and localized prostate cancer, and a lack of correlation between mtDNA levels and clinical or pathological variables such as PSA, Gleason score and lymph node invasion [11,20]. However, one study reported correlations between PSA levels and both total mtDNA and total mtRNA levels in the plasma of prostate cancer patients, with mtDNA levels 2.5-fold higher in the plasma of prostate cancer patients compared with benign controls [19]. Interestingly, mtDNA appears to be of greater prognostic than diagnostic utility in prostate cancer serum/plasma, particularly in advanced prostate cancer. For example, an increase in short mtDNA fragments was described in patients with early PSA recurrence following radical prostatectomy [11], and mtDNA is associated with increased prostate cancer-specific mortality [19]. Indeed, patients who did not survive to 2-year follow-up had 2.6-fold higher circulating mtDNA level at initial presentation than surviving patients, and 2-year survival for patients with high circulating mtDNA was 35% compared with 73% for patients without elevated circulating mtDNA [19].

Jeronimo *et al.* sequenced the D-loop region, 16S rRNA and complex I of mtDNA in primary prostate tumors and in patients' urine and plasma, to investigate whether mtDNA is mutated in prostate cancer. Twenty mtDNA mutations were described in primary tumors, and where mtDNA mutations were identified in plasma, these were also found in primary tumors of affected patients [18]. However, such mutations were a relatively rare event, with mtDNA mutations identified in only three of 16 patients examined [18], limiting the diagnostic potential of such mutations.

Circulating mRNAs as biomarkers

The utility of circulating mRNAs as biomarkers is hampered by the low specificity of qPCR-based assays, and use of target mRNAs that are prostate-specific, but not always prostate cancer-specific. For example, circulating PSA mRNA is detected after prostate biopsy of healthy individuals as well as prostate cancer patients [51,52]. In addition, circulating mRNA is less stable than circulating DNA, resulting in lower abundance of mRNA targets for qPCR applications. Thus, standard qPCR-based assays of circulating tumor-related mRNAs from serum/plasma seem of limited value as diagnostic biomarkers for prostate cancer. That said, circulating mRNAs have demonstrated potential for distinguishing patients with organ-confined disease from those with metastatic disease. *BMP6* expression has been demonstrated to be high in primary tumors of patients with metastatic prostate cancer and low or undetectable in individuals with localized, nonmetastatic prostate cancer and in benign prostate tissue, and appears to play a key role in promotion of bone metastasis by enhancing osteoblastic and invasive capabilities of prostate cancer cells [53,54]. For this reason, Deligezer *et al.* compared levels of post-treatment circulating BMP6 mRNA between patients with local, locally advanced and metastatic prostate cancer using qPCR [50]. It was found that levels of BMP6 mRNA were significantly higher in patients with metastatic prostate cancer compared with local or locally advanced disease [50]. In addition, BMP6 plasma mRNA levels were able to correctly identify metastatic prostate cancer cased from localized or locally advanced prostate cancers in 71% of cases [50]. These data provide

evidence as to the utility of plasma BMP6 mRNA levels, in combination with PSA, as an indicator of disease progression and/or treatment response. In the future, upon identification of additional biomarkers that discriminate metastatic from nonmetastatic disease, BMP6 plasma mRNA levels may form one component of a biomarker panel test to identify micrometastatic prostate cancer at time of diagnosis.

Telomerase activity has been demonstrated to be increased in 85–100% of human cancers compared with benign tissues [55], and hTERT mRNA has been detected in prostate cancer patients' plasma/serum [56,57]. Thus, plasma hTERT mRNA levels were investigated for their diagnostic accuracy, ability to predict biochemical recurrence and correlation with clinicopathological features in a study of 105 patients with elevated PSA and 68 healthy controls [58]. As a diagnostic biomarker, plasma hTERT mRNA demonstrated increased sensitivity, specificity, positive predictive value and negative predictive value compared with serum PSA. It was also found that plasma hTERT mRNA correlated significantly with clinicopathological indicators of poor prognosis, and is a highly significant independent predictor of prostate cancer diagnosis and biochemical recurrence, unlike PSA [58]. In addition, patients with high levels of plasma hTERT mRNA demonstrated reduced recurrence-free survival compared with those with low levels, an effect not observed for plasma PSA [58], although it should be noted that only seven patients with biochemical recurrence were included in this study. Together these data suggest that plasma hTERT mRNA levels may offer greater diagnostic and prognostic value than PSA, and that, combined with other markers, it may offer a highly accurate, noninvasive biomarker for prostate cancer diagnosis.

AGR2 mRNA may also have a role as a potential biomarker for prostate cancer. The protein product of this gene is associated with metastatic progression and cell migration in prostate cancer cells, and urine AGR2 levels have been investigated as a putative diagnostic prostate cancer biomarker [59,60]. Kani *et al.* measured AGR2 mRNA levels in the plasma of patients with hormone-sensitive prostate cancer, castrate-resistant prostate cancer (CRPC) and neuroendocrine-predominant CRPC (NP-CRPC) by qPCR. It was demonstrated that AGR2 mRNA levels are significantly elevated in patients with metastatic prostate cancer, and are highest in patients with clinicopathological indicators of NP-CRPC [61]. As PSA levels are frequently not elevated in patients with NP-CRPC, PSA cannot be used as a therapeutic response marker, or to aid diagnosis, in these tumors, which have very poor prognosis. However, given that AGR2 levels are raised in the plasma of such patients, the authors suggest that AGR2 mRNA levels may be used as an aid to noninvasively identify patients with NP-CRPC and to subsequently assist with treatment planning [61]. Given that these proposals are based on the data of only three patients with NP-CRPC, far larger studies will be required to ascertain the clinical usefulness of AGR2 mRNA as a NP-CRPC diagnostic biomarker.

Circulating miRNAs as biomarkers

miRNAs are naturally occurring single-stranded RNA molecules, 19–25 nucleotides in length, capable of post-transcriptional regulation of target mRNAs to which they bind, at complementary sequences most frequently in the 3′-untranslated region [62]. Reduced

levels of the encoded protein result from subsequent translational repression or mRNA degradation. Furthermore, miRNAs can function as either oncogenes, encouraging tumor growth, or tumor suppressors, repressing it – collectively termed oncomirs (as reviewed in Heneghan *et al.* [63]). Indeed, there are reports of a given miRNA having both these effects, for example miRNA-125b has oncogenic activity in prostate cancer but acts as a tumor suppressor in ovarian and breast cancer [64]. This is likely due to the fact that miRNAs have pleiotrophic effects since each can potentially target hundreds of transcripts, hence the overall function of a given miRNA in a particular context is determined by the relative availability of the target mRNAs.

The desirable properties of miRNAs in the context of circulating biomarkers include stability (they are stable even in archival samples) and availability (they have been isolated from most body fluids) [64]. Tumor cells release miRNAs into the blood and circulating expression profiles of miRNAs are altered in many tumor types, suggesting that the miRNA profile can be informative about the disease [63,65]. Furthermore, detection and quantitation can be relatively easily achieved in low volumes of blood serum or plasma qPCR, which is both specific and sensitive [66].

miRNAs as diagnostic biomarkers

Recently, there has been much discussion as to the actual benefit of PSA as a biomarker to screen populations. Two large multinational randomized prospective control trials, the ERSPC and the PLCO studies, were conducted in this area. They found that PSA screening did not provide any substantial benefit in overall patient survival [2]. Following these results the United States Preventative Services Task Force (USPSTF) recommended that the population benefit of PSA screening is inconclusive and does not recommend it for men of any age [67]. There is, therefore, a particular need for new, specific diagnostic biomarkers that define populations of men with prostate cancer needing treatment, rather than indolent cancer that can be monitored without treatment.

The most widely researched area for miRNAs as biomarkers has been in the search for a new diagnostic test. The first report was by Mitchell *et al.* in 2008, who probed a panel of miRNAs in the serum of healthy men and those with advanced prostate cancer, and found that miR-141 was highly elevated in the serum of the men with prostate cancer. In addition, miR-141 levels correlated significantly with serum PSA levels and could differentiate individuals with advanced prostate cancer with 60% sensitivity and 100% specificity [68]. However, this low level of accuracy would mean that 40 out of every 100 men with raised miR-141 would have an unnecessary biopsy.

In recent studies Moltzahn *et al.* looked in the serum of 36 early-stage prostate cancer patients immediately prior to prostatectomy compared with the serum of 12 healthy men [69]. Receiver operated curves for individual miRNAs showed that several possessed significant diagnostic capability. Three miRNAs – miR-93, miR-106a and miR-24 – showed consistently elevated levels in the high-risk group according the Cancer of the Prostate Risk Assessment (CAPRA) score (score >5 with lymph node-positive disease) when compared with the healthy controls. In other studies, Bryant *et al.* used high-throughput qPCR profiling

and found 12 miRNAs to be altered in the circulation of 78 men with prostate cancer compared with 28 healthy men, with miR-107 having the greatest fold change [70], and Chen *et al.* showed that a five-miRNA panel (in which let-7e, let-7c and miR-30c were downregulated and miR-622 and miR-1285 were upregulated) yielded an area under the curve (AUC) of 92% in discriminating prostate cancer from BPH [71].

Some miRNA species have been highlighted as putative biomarkers across multiple studies, notably miR-141. Yaman Agaoglu *et al.* tested the diagnostic utility of three miRNAs, miR-21, miR-141 and miR-221. The authors found miR-21 and miR-221 to be elevated in the serum of 18 men with localized prostate cancer when compared with the 20 healthy controls, and found that miR-141 could distinguish men with bone metastases from those with localized disease [72]. A study by Mahn *et al.* highlights the importance of age-matched controls. In this study, miR-26a, miR-195 and let-7i were increased in the serum of men with localized prostate cancer as compared with BPH patients. However, this difference was not evident when serum levels from the same prostate cancer patients were compared with those in healthy men [73]. A likely explanation is that these miRNAs are altered with age and/or hormonal state. In prostate cancer this may be particularly relevant given the median age of the patient population and the domination of the androgen receptor pathway in the mechanism of disease. Some miRNAs associated with prostate cancer, namely miR-141 and miR-27a, are known to be under androgen control; therefore, their expression levels will be affected by hormonal therapies as well as the disease state [74,75]. See Table 1 for a summary of miRNAs of interest in diagnosis.

miRNAs as prognostic biomarkers

Unfortunately, there is, as yet, no reliable method to differentiate patients with cancer that will progress to life-threatening disease from the majority whose cancers take a more indolent course – approximately 25% of men treated with curative intent for localized prostate cancer experience relapse within 5 years [76]. Distinguishing those patients likely to relapse and therefore requiring postoperative consolidating treatment, such as radiotherapy or androgen blockade, is based on an evaluation of a collection of markers, including PSA, Gleason score and histological score. These are collated into a surrogate prognostic biomarker algorithm such as the D'Amico or the CAPRA score [77], which can then be used to guide treatment choices – but like the PSA test, they lack specificity and sensitivity. If it was possible to identify this subset with a robust prognostic biomarker, a majority of men could be spared from significant morbidity associated with the side effects of 'excessive' prostate cancer treatment.

Prognostic miRNA markers have been sought using two study designs. The first was to identify miRNAs elevated in metastatic disease and then to measure these in localized disease to see if they could identify the subset of patients with poor prognostic indicators [78]. Brase *et al.* initially found 69 miRNAs to be elevated in men with metastatic disease; subsets of these were then measured in men with localized prostate cancer [78]. Three miRNAs – miR-141, miR-200b and miR-375 – were found to be elevated with increasing tumor stage and Gleason score. This design was also adopted by Nguyen *et al.*, who confirmed that miR-141 and miR-375 can distinguish patients with metastatic prostate

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cancer from those with low-risk localized cancer [79]. The second experimental strategy compared miRNA levels in patients divided by D'Amico or CAPRA score, such as the study conducted by Moltzahn *et al.*, who found miR-24 levels were lower in the cohort of patients with a higher CAPRA score, while higher levels of miR-106a, miR-451 and miR-93 were found in the cohort of patients with a lower CAPRA score [69]. In a similar study, Shen *et al.* found miR-20a and miR-21 were significantly increased in patients with a high-risk CAPRA score [80].

From these prognostic biomarker studies, miR-141 and miR-375 in particular emerge as significant disease correlates. In the future these could potentially be used in a test to identify patients with previously undetectable micrometastases at the time of diagnosis. Further studies are needed to validate candidate prognostic miRNAs in larger cohorts with long-term clinical follow-up. See Table 2 for a summary of miRNAs of interest in prognosis of prostate cancer.

miRNAs as predictive biomarkers

Predictive markers are defined as those that can be used to predict a patient's response to a given drug or therapy, and thus can be used to stratify patients for treatment. There are currently no useful predictive biomarkers in prostate cancer management. Treatments for prostate cancer such as docetaxel chemotherapy only work (i.e., reduce symptoms or prolong survival) in a proportion of cases but cause symptoms and morbidity at some level for all patients receiving such therapy, hence predictive biomarkers have the potential to significantly increase quality of life for patients by reducing treatment-induced morbidity. In one study, Zhang *et al.* found that in a small cohort of docetaxel-resistant, castrateresistant prostate cancer patients there were significantly higher circulating miR-21 levels than in patients who responded to the drug [81]. In addition, levels of this miRNA were significantly higher in CRPC and androgen-dependent prostate cancer in patients with PSA > 4 ng/ml. As well as predicting response to a given therapy, such miRs could also provide information as to the mechanisms of resistance to a given therapy.

Conclusion

A number of cell-free DNAs and miRNAs represent promising prostate cancer biomarkers but they require extensive and detailed standardization and confirmation of clinical utility in large, multicentered studies with extensive followup. It is likely that no single cell-free DNA or miRNA will be robust enough to form a clinically useful tool; however, as part of marker panels potentially used in combination with PSA, they will hopefully prove their worth by improving diagnostic efficiency and facilitating patient stratification to improve targeting of appropriate treatments to patients who will benefit.

Future perspective

Circulating nucleic acids represent a large population of very promising potential biomarkers, but significant challenges remain regarding their use as biomarkers in the clinic. In addition to screening studies, it is vital that there is more basic scientific research to

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elucidate the role and function of these circulating nucleic acids. This will inform selective and guided miRNA screening to interrogate the extensive tumor banks available. Some of the miRNAs discussed herein are poised to go into clinical testing on larger cohorts, so we keenly anticipate the development of new predictive tests based on these, and in turn potentially a decrease in surgical intervention and associated morbidity.

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Executive summary

The need for new biomarkers for prostate cancer

- Prostate cancer is the most prevalent cancer in the UK.
- Prostate cancer needs a new biomarker.
- Currently, PSA is the marker of choice for screening patients for cancer of the prostate. Two large independent clinical trials have recently highlighted the problems with this as a biomarker.

Cell-free DNAs as biomarkers

- Cell-free DNA alterations in prostate cancer are potential biomarkers. These include gene promoter hypermethylation, mitochondrial DNA mutations and microsatellite instability.

Circulating mRNAs as biomarkers

- Detection of circulating mRNA is of limited value owing to the low stability of RNA and the low specificity of the real-time PCR-based assays.

Circulating miRNAs as biomarkers

- miRNA studies show promise but require standardization of methodology and robust validation to elucidate reliable candidate biomarkers.

Conclusion

- It is likely that a combination of circulating nucleic acid markers will form a biomarker panel in combination rather than one individual marker.

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Table 1

Diagnostic miRNAs of interest.

Study (year)	miRNA	Direction of change	Study design	Ref.
Chen *et al.* (2012)	Let-7c	↓	PC vs BPH and HCs	[71]
Chen *et al.* (2012)	Let-7e	↓	PC vs BPH and HCs	[71]
Mahn *et al.* (2011)	Let-7i	↑	mPC vs BPH	[73]
Agagolu *et al.* (2011)	miR-21	↑	PC vs HCs	[72]
Moltzahn *et al.* (2011)	miR-24	↓	PC vs HCs	[69]
Mahn *et al.* (2011)	miR-26a	↑	mPC vs BPH	[73]
Chen *et al.* (2012)	miR-30c	↓	PC vs BPH and HCs	[71]
Moltzahn *et al.* (2011)	miR-93	↑	PC vs HCs	[69]
Moltzahn *et al.* (2011)	miR-106a	↑	PC vs HCs	[69]
Bryant *et al.* (2012)	miR-107	↑	PC vs HCs	[70]
Mitchell *et al.* (2008)	miR-141	↑	mPC	[68]
Brase *et al.* (2011)	miR-141	↑	mPC vs localized	[78]
Nguyen *et al.* (2013)	miR-141	↑	HRPC vs localized	[79]
Mahn *et al.* (2011)	miR-195	↑	mPC vs BPH	[73]
Yaman Agaoglu *et al.* (2011)	miR-221	↑	PC vs HCs	[72]
Brase *et al.* (2011)	miR-375	↑	mPC vs localized	[78]
Nguyen *et al.* (2013)	miR-375	↑	HRPC vs localized	[79]
Nguyen *et al.* (2013)	miR-378	↑	HRPC vs localized	[79]
Nguyen *et al.* (2013)	miR-409-3p	↑	HRPC vs localized	[79]
Chen *et al.* (2012)	miR-622	↑	PC vs BPH and HCs	[71]

↑: Increased expression; ↓: Decreased expression; BPH: Benign prostatic hypertrophy; HC: Healthy control; HRPC: Hormone-refractory prostate cancer; mPC: Metastatic prostate cancer; PC: Prostate cancer.

Table 2

Prognostic miRNAs of interest.

Study (year)	miRNA	Study design	Ref.
Shen *et al.* (2012)	miR-20c	Correlated with tumor stage and D'Amico score	[80]
Shen *et al.* (2012)	miR-21	Correlated with CAPRA score and D'Amico score	[80]
Moltzahn *et al.* (2011)	miR-24	Decreased with increasing CAPRA score	[69]
Moltzahn *et al.* (2011)	miR-106a	Correlated with increasing CAPRA score	[69]
Moltzahn *et al.* (2011)	miR-93	Correlated with increasing CAPRA score	[69]
Brase *et al.* (2011)	miR-141	Correlated with Gleason score and lymph node status	[78]
Gonzales *et al.* (2011)	miR-141	Correlated with CTCs, PSA and LDH	[82]
Shen *et al.* (2012)	miR-145	Correlated with D'Amico score	[80]
Brase *et al.* (2011)	miR-200b	Correlated with tumor stage and Gleason score	[78]
Shen *et al.* (2012)	miR-221	Correlated with D'Amico score	[80]
Brase *et al.* (2011)	miR-375	Correlated with Gleason score and lymph node status	[78]
Nguyen *et al.* (2013)	miR-375	Increased in HRPC vs localized PC	[79]
Nguyen *et al.* (2013)	miR-378	Increased in HRPC vs localized PC	[79]
Nguyen *et al.* (2013)	miR-409–3p	Increased in HRPC vs localized PC	[79]
Moltzahn *et al.* (2011)	miR-453	Correlated with increasing CAPRA score	[69]

CAPRA: Cancer of the Prostate Risk Assessment; CTC: Circulating tumor cell; HRPC: Hormone-refractory prostate cancer; PC: Prostate cancer.

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