

## REVIEW ARTICLE

## Identification of Biomarkers for Prostate Cancer

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## Abstract

**BACKGROUND:** Prostate cancer (PCa) was the second most common type of cancer and the fifth leading cause of cancer-related death in men. The great challenge for physicians is being able to accurately predict PCa prognosis and treatment response in order to reduce PCa-specific mortality while avoiding overtreatment by identifying of when to intervene, and in which patients.

**CONTENT:** Currently, PCa prognosis and treatment decision of PCa involved digital rectal examination, Prostate-Specific Antigens (PSA), and subsequent biopsies for histopathological staging, known as Gleason score. However, each procedure has its shortcomings. Efforts to find a better clinically meaningful and non-invasive biomarkers still developed involving proteins, circulating tumor cells, nucleic acids, and the ‘omics’ approaches.

**SUMMARY:** Biomarkers for PCa will most likely be an assay employing multiple biomarkers in combination using protein and gene microarrays, containing markers that are differentially expressed in PCa.

**KEYWORDS:** prostate cancer, PSA, biomarkers, nomograms, miRNA, proteomic, genomic, metabolomic

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## Abstrak

**LATAR BELAKANG:** Kanker prostat / *prostate cancer* (PCa) merupakan kanker kedua yang paling sering ditemukan, dan menduduki urutan kelima sebagai penyebab kematian pada pria. Tantangan terbesar bagi para dokter adalah bagaimana dapat memprediksi dengan akurat prognosis dan respon terapi PCa dengan tujuan untuk mengurangi kematian akibat PCa, tanpa memberikan terapi yang berlebihan, serta mampu mengidentifikasi waktu intervensi yang tepat pada pasien yang tepat.

**ISI:** Saat ini, prognosis dan terapi PCa dilakukan atas dasar pemeriksaan rektal digital, *Prostate-Specific Antigens* (PSA), dan biopsi untuk mengetahui derajat histopatologisnya, yang dikenal sebagai skor Gleason. Bagaimanapun, setiap prosedur memiliki keterbatasannya. Usaha untuk menemukan biomarker yang bermanfaat secara klinis dan tidak invasif masih terus dilakukan menggunakan protein, sel tumor di sirkulasi, asam nukleat, dan pendekatan “omics”.

**RINGKASAN:** Biomarker PCa akan berupa suatu kombinasi biomarker, meliputi protein dan *microarray* gen yang berisi banyak marker yang diekspresikan pada PCa.

**KATA KUNCI:** kanker prostat, PSA, biomarker, nomogram, miRNA, *proteomic*, *genomic*, *metabolomic*

## Introduction

Prostate cancer (PCa), also known as carcinoma of the prostate, is the development of cancer in the prostate, a

gland in the male reproductive system. Globally, PCa was the second most common type of cancer and the fifth leading cause of cancer-related death in men. It was estimated about 233,000 new cases and 29,480 deaths from PCa in the United States in 2014, and 9,033 annual incidences with

6,841 mortality cases in Indonesia until year 2008.(1,2,3)

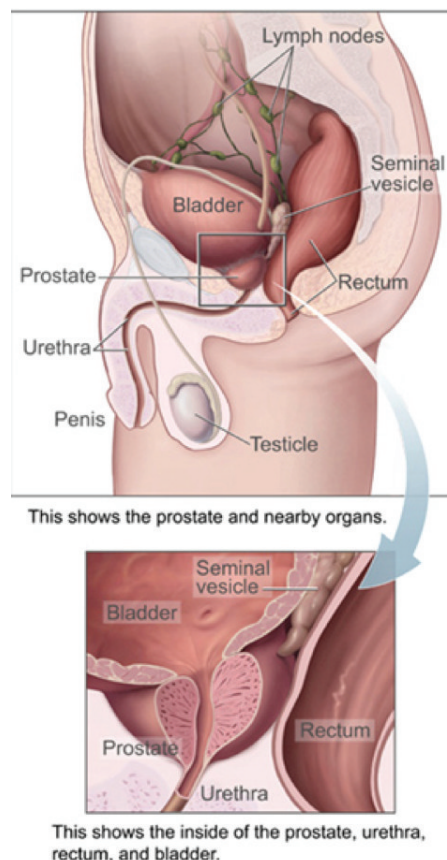
PCa is very uncommon in men younger than 45, but becomes more common with advancing age. The average age at the time of diagnosis is 70.(4) However, many men never know they have PCa, this was because early PCa usually causes no symptoms, or often have symptoms similar to any prostatic diseases such as benign prostatic hyperplasia, or prostatitis. These include frequent urination, nocturia (increased urination at night), difficulty starting and maintaining a steady stream of urine, hematuria (blood in the urine), and dysuria (painful urination).(5,6)

The great challenge for physicians is being able to accurately predict PCa prognosis and treatment response in order to reduce PCa-specific mortality while avoiding overtreatment by identifying of when to intervene, and in which patients.(7) Although risk stratification using Prostate-Specific Antigens (PSA), Gleason grading and T stage have helped tremendously in determining if active surveillance is an appropriate option and in defining the optimal treatment for localized PCa (8), recent data suggest that many men with localized PCa, even of higher grade, do not enjoy a survival benefit from treatment (9). Thus, a large number of men are subjected to toxicities of treatment such as radiation or surgery with resulting decrement in quality of life with no potential benefit. The ability to predict with biomarkers which local therapy could provide the best chance of disease control and the patients susceptibility to toxicities of certain treatments would provide another chance to improve disease control outcomes and predict for toxicity from radiation.(10,11)

## Pathophysiology of PCa

Prostate is a part of the male reproductive system that helps make and store seminal fluid, about 3 centimeters long and weighs about 20 grams in adult men contains many small glands which make about 20 percent of the fluid constituting semen. The prostate located in the pelvis, under the urinary bladder and in front of the rectum. It surrounds part of the urethra, the tube that carries urine from the bladder during urination and semen during ejaculation. That's why prostate diseases often affect urination, ejaculation, and rarely defecation.(12-14)

PCa happened when the cells of these prostate glands mutate into cancer cells. The growth of PCa depends on the ratio of cells proliferating to those dying. Androgen are the main regulator of this ratio between cell proliferation stimulating and apoptosis. This called androgen-dependent PCa (16). Somehow, a general increase in the mutation rate



**Figure 1. Anatomy of prostate.** (15) (Adapted with permission from Wikipedia).

would then increase the likelihood of a cell developing ensuing mutations ('multiple hits') that allow PCa cell to grow independently of androgen (androgen-independent PCa) (AIPC). There are many proposed mechanisms how the PCa can develop into AIPC (see Table 1).

The primary causes and the molecular mechanisms underlying of PCa are poorly understood. This is the reason why there are no effective prevention strategies or treatment modalities to cure advanced PCa. Several aspects suggested involved in PCa development including: [1] higher oxidants and electrophiles exposure, both exogenous (environmental toxins and dietary) and endogenous (cellular metabolism, mitochondrial dysfunction, hypoxia) in individual with PCa. It was well-known that oxygen radicals can directly attack DNA, promoting chronic oxidative stress which results in lipid peroxidation and generation of wide range of other reactive products with the potential to damage DNA (18,19), [2] Phagocytic inflammatory cells release reactive oxygen and nitrogen species in attempts to eradicate infectious organisms (or perceived infectious organisms). Repeated bouts of this immune-mediated oxidant and nitrogenous injury over many years developed chronic inflammation and could play a major role in the pathogenesis of cancer (20). [3] Focal prostatic gland atrophy occurs primarily in

**Table 1. Mechanisms of development of AIPC.**(17) (adapted with permission from Nature Publishing Group).

Type	Pathway	Ligand dependence	AR dependence	Mechanism
1	Hypersensitive AR	Androgen dependent	AR dependent	Amplified AR Sensitive AR Increased DHT
2	Promiscuous AR	Pseudo-androgens Androgen antagonists Corticosteroids Coregulator mutations	Dependent on a mutant AR in LNCaP cells and AR <sup>CR</sup> cells	Widened AR specificity Illicit stimulation by non-androgens 'Flutamide withdrawal' (antagonists acting as agonists)
3	Outlaw AR	Androgen independent Ligand independent	AR dependent	Mutant <i>PTEN</i> Amplified <i>HER-2/neu</i> Activated PI3K Activated MAPK Mutant coregulators
4	Bypass AR	Androgen independent	AR independent	Parallel or alternative survival pathways: • Overexpression of <i>BCL2</i> • Activation of other oncogenes • Inactivation of tumour suppressor genes
5	Lurker cells	Androgen independent	AR independent	Malignant epithelial stem cells

AR, androgen receptor; AR<sup>CR</sup>, cortisol and cortisone responsive, AR; DHT, dihydrotestosterone; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase.

the outer part of the gland, referred to as the peripheral zone, the preferred site for carcinoma, because most focal prostate lesions appear to be proliferative rather than quiescent. The proliferation is increased, while apoptosis is not (21-23). [4] The telomere shortening contribute in genetic instability and develop PCa (24). These understanding of PCa developing mechanisms was expected to be the foundation for researcher and clinicians to find better biomarkers for diagnosing PCa in early stage, can accurately predict and monitor the tumor progression, and the response to treatments.

## Identification of Biomarkers for PCa

Accurate and timely assessment of PCa prognosis remains as one of the most challenges in PCa management. Rapid advances in molecular technology, and overwhelming number of proposed biomarkers nowadays still can not prevent many over-diagnosed of PCa and many patients are treated in an unnecessarily aggressive manner. Possible reasons are the complex nature of this disease.(25) Currently, PCa prognosis and treatment decision involved digital rectal examination, PSA, and subsequent biopsies for histopathological staging, known as Gleason score.(26) However, each procedure has its shortcomings, and here, we will summarize some promising biomarkers for PCa.

A biomarker is a measurable biological indicator that can provide information about the presence or progression of a disease or the effects of a given treatment. A clinically useful biomarker should be safely obtainable from the

patient by non-invasive means, have high sensitivity and specificity, high positive and negative predictive values, and facilitate clinical decisions that allow optimal care to be administered.(27)

## PSA

PSA or human kallikrein-related peptidase 3 (hKLK3) is a 33 kDa glycoprotein of the kallikrein family, encoded by the *hKLK3* gene located in the long arm of chromosome 19 within the region spanning q13.2–q13.4. In normal prostate, PSA is secreted from the prostatic epithelium into the secretory ducts to contribute to the seminal fluid. In PCa, disruption of the basal-cell layer allows PSA to “leak” into the circulation resulting in elevated serum levels of PSA. Therefore, it is organ specific and not disease specific.(28-30) PSA also can be elevated in other benign conditions of the prostate. This makes PSA not specific to PCa though it has been regarded as the best cancer biomarker due to its high sensitivity.(31) This high false - positive rates increasing the risk of patients’ overdiagnosed and having unnecessary treatment or surgery, while insignificantly decreasing the mortality due to PCa.(32) Positive predictive values for PSA have shown it to operate at 37%, with 25% of men in the ‘gray zone’ (4–10 ng/ml) having PCa (33) and 15% of individuals with PSA concentrations  $\leq$ 4 ng/ml having PCa (34).

To increase its accuracy, several methods of measuring PSA have been developed that include: monitoring personal PSA changes over time (PSA velocity); the ratio of PSA to prostate volume (PSA density) determined by transrectal

ultrasound; and PSA ranges that are specific to age, measuring the splice isoforms and complexed forms of PSA (free PSA (fPSA) versus total PSA (tPSA)) which shown to have a predictive value for late-stage PCa, and help discriminate between PCa and Benign Prostatic Hypertrophy (BPH) for men with “gray zone” PSA.(35,36) PSA in circulation has also been found to be complexed to other binding proteins and this measurement has shown to add clinical utility. These include PSA bound to  $\alpha$ 2-macroglobulin,  $\alpha$ 1-antichymotrypsin and  $\alpha$ 1-protease inhibitor. In addition, there are several post-translationally modified cleavage isoforms of PSA that have been measured specifically.(37)

### Gleason Score

Currently, Gleason grading is considered to be the best predictor of outcome. The Gleason score is a number derived from the biopsy specimen a pathologist sees under a microscope, based on the degree of loss of normal glandular tissue.(38) Pathologist will look for the most prominent cell type, (the primary Gleason grade), then the second most prominent cell type (the secondary Gleason grade). The numerical grade range from 1 to 5. The sum of the primary grade plus the secondary grade equals the Gleason score. Patients with Gleason scores 7 or higher are at increased risk of extraprostatic extension and recurrence after therapy. (8,39).

The multifocal nature of PCa, whereby different genetic alterations may exist in different tumor foci of a prostate, however, increases the likelihood of missing a high-grade focus. Furthermore, the risks associated with biopsies, such as bleeding and increased risk of infections potentially leading to sepsis, underscore the need for alternative approaches for accurate prognosis.(40)

### hKLLK2

hKLLK2 is a serine protease enzyme from the kallikrein family of serine proteases, the same gene family as PSA, and shows 80% sequence homology with PSA, although its enzymatic activities differ.(41,42) Tissue expression of KLK2 has been shown to correlate well with PCa progression and tumor volume and has been studied as a peripheral marker in serum in combination with PSA and fPSA.(43-45) KLK2 has also been shown to have independent clinical utility as a prognostic indicator for biochemical recurrence in men with PSA  $\leq$ 10 ng/ml.(46)

### Prostate Cancer Antigen 3 (PCA3)

PCA3, also known as Differential Display Code 3 (DD3), is a noncoding RNA that specifically expressed in the prostate and highly expressed in over 90% of PCa tumors compared

with BPH specimens.(47-49) PCA3 can be detected in urine and prostatic fluid.(50) A ratio of the PCA3:PSA RNA, known as the PCA3 score, is used, in combination with other clinical information, to guide decisions on repeat biopsy in men who are 50 years of age or older and who have previously had at least one negative prostate biopsy (PBX).(51)

### Nomograms for Predicting PCa

According to current European Association of Urology (EAU) guidelines, the need for PBX should be further determined on consideration of patient’s biological age, potential comorbidities (American Society of Anesthesiologists [ASA] Index and Charlson Comorbidity Index), and the therapeutic consequences (risk stratification), to prevent a significant proportion of men from being exposed to unnecessary procedures and associated psychological distress.(52)

To improve prediction of PBX outcome and better counsel patients either to undergo or forgo PBX, statistical models have been developed that combine the strengths of several clinical variables. There are different forms of prediction models, for example, nomograms or risk calculators. Risk calculators are based on logistic regression, resulting in a risk score to support clinical decision-making for PBX.(53-55) Ideally, a nomogram should be capable of identifying PCa at PBX without missing men with high-grade PCa, and preventing a significant proportion of men without, or with insignificant, PCa from undergoing PBX. The intention is to reduce disease morbidity and mortality by detecting significant PCa at an early stage, and at the same time to avoid overdiagnosis as well as overintervention.(56)

Nomogram prediction can never be perfect, which is shared with all prognostic models and is mainly due to lack of consideration of all predictive risk factors and the inability to assemble all known prognostic factors optimally. (57) Some tests that may have the potential to hold up to their promise when it comes to prediction of PCa risk are the Prostate Health Index (PHI), PCA3 and a human kallikrein panel.(56)

The PHI is a new formula that combines all three PSA isoforms (tPSA, fPSA and [-2]proPSA or p2PSA) into a single score that can be used to aid in clinical decision-making.(58) PHI is calculated using the following formula:  $(p2PSA/fPSA) \times \sqrt{tPSA}$ . Intuitively, this formula makes sense, men with a higher tPSA and p2PSA with a lower fPSA are more likely to have clinically significant PCa.(59) Combined serum hKLLK2 to three other kallikreins (tPSA, fPSA and intact PSA) called as the ‘four kallikrein panel’ demonstrated improved predictive accuracy of PBX outcome

in men with elevated tPSA levels. Predictive accuracy increased from 72 to 84% in an external validation cohort, leading to a reduction of unnecessary biopsies (60). Only four urinary PCA3-based nomograms have been previously published, mostly combining patients' age, Digital Rectal Examination (DRE), PSA, fPSA, sampling density and PCA-3. Two are proposed to all patients, whatever the medical history of previous biopsies, and were externally validated: the updated version of the PCa Prevention Trial (PCPT) risk calculator and the graphically available nomogram published by Chun *et al.*(61-63) Another is specifically dedicated to patients scheduled for repeat biopsy (64), while the last one, very recently published by Hansen *et al.* (65), has been developed for guiding the initial biopsy decision. Both Hansen's and Chun's nomograms proved to provide significant clinical benefit without missing a too important proportion of high-grade PCa (HGPCa).(62,65)

### **Homeobox-containing Transcription Factor Engrailed-2 (EN2)**

The Homeobox gene family incorporates over 100 members, which each encode a homeo-domain-containing protein, this domain itself being a 61 amino acid protein. This specific domain acts as a binding site for other proteins to enable activation or repression of downstream target genes. EN2 is a homeobox-containing transcription factor secreted specifically by PCA into urine, where it can be detected by a simple ELISA assay.(66)

EN2 was originally identified as a potential oncogene in breast cancer, as forced overexpression of the gene promoted malignant characteristics in mammary cell lines (67), but then hypermethylation of EN2 has also been identified in several cancers, including lung and astrocytoma, although its specific role is yet to be characterized.(68,69) EN2 protein expression first confirmed in PCa tissue (and the absence of EN2 in normal prostate or non-cancer prostatic disorders) in 2011.(70) Secretion and deposition of EN2 protein into urine by men with PCa was hypothesized and subsequently confirmed by western blot analysis of urinary supernatant. An ELISA test has been developed for the accurate quantitation of urinary EN2, and a point of care test has been developed and is being evaluated.(70)

Many studies showed the potential utility of urinary EN2 not only as a diagnostic biomarker for PCa, but also as an accurate indicator of PCa volume. Noninvasive measures of cancer volume will be extremely useful in aiding the urologist to offer radical treatment versus advising an active surveillance approach. The EN2 test is a robust, simple, low-cost urine-based test. EN2 protein in urine is stable for at least 4 days at room temperature allowing

patients samples to be collected and transported routinely at low cost. However, there are a number of remaining unresolved issues. These include the need to understand the EN2 expression regulation and secretion by cancer cells, determining whether the cut-off level of 42 ng/ml is optimal, the correlation of EN2 with tumor grade, defining the role of EN2 in monitoring patients after radiotherapy or hormonal therapy and, probably most immediately, whether EN2 secretion in some way can be used in conjunction with serum PSA to improve diagnostic efficacy (71).

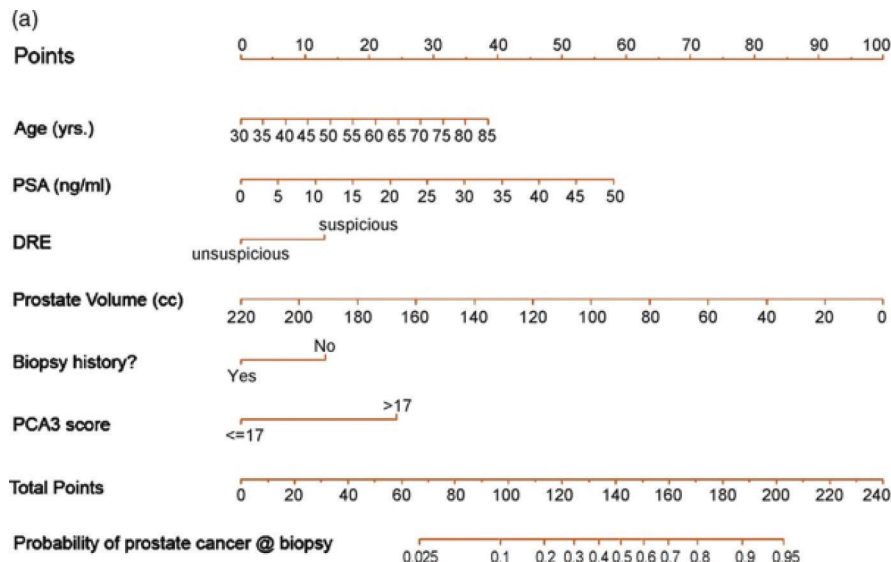
### **ETS-related Gene (ERG)**

Transmembrane protease, serine 2 (TMPRSS2) and E26 transformation-specific (ETS) transcription factors fusions in PCa were initially discovered by cancer profile outlier analysis to be present in 80% of prostate tumors studied.(43) Since this initial discovery, many other similar gene fusions have been discovered associated to PCa.(72) Tomlins *et al.* (73) first reported the occurrence of a recurrent TMPRSS2-ERG fusion transcript in those with prostate tumors. These fusions were detectable in 42% of urinary expressed prostatic secretion samples from men with prostate cancer, and less in prostatic intraepithelial neoplasia (PIN) and BPH tissues (74). Another area in which the clinical utility of the cancer-specific TMPRSS2-ERG fusion product is currently being extensively investigated is urine-based detection in a preoperative setting for early diagnosis of PCa and, potentially, to distinguish indolent versus aggressive disease (75-78). The urine assay measures TMPRSS2-ERG mRNA relative to PSA mRNA (TMPRSS2-ERG score) in post-digital rectal exam urine.(75,76)

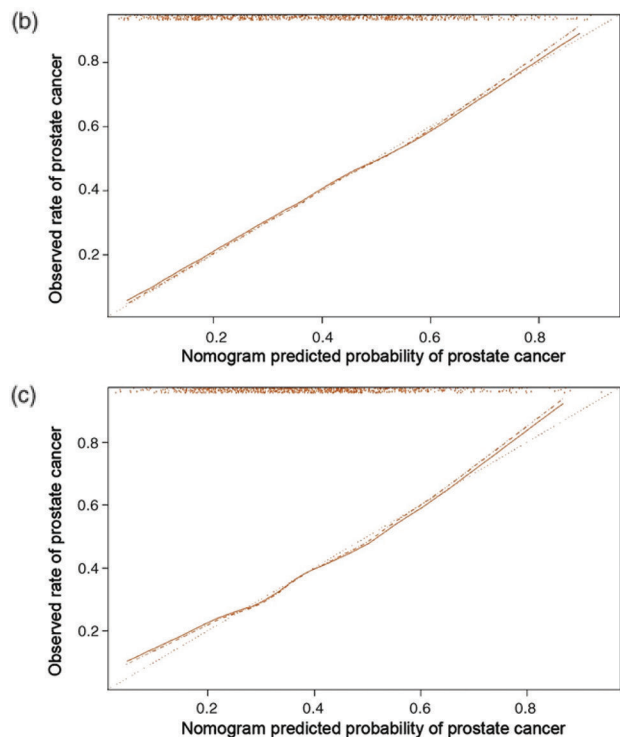
Two important limitations of ERG was critical to be aware are: first, the intertumoral (between different cancer foci) heterogeneity of ERG expression might limit its use in a preoperative (biopsy) setting; second, the molecular heterogeneity of PCa arising from different zones of the gland impacts on the prevalence of ERG rearrangement status. Then, the combination of TMPRSS2-ERG score with another multiple urine biomarker may increase the diagnostic and prognostic value of single assays and may reduce the number of prostate biopsies performed in the future.(79)

### **Urinary Biomarkers: $\alpha$ -Hepatocyte Growth Factor ( $\alpha$ HGF), Insulin-like growth factor-binding protein 3 (IGFBP3), and Osteopontin (OPN)**

Protein urinary markers have more potential for functionally interrogating the tumor, as prostatic products are directly secreted into the urinary tract, theoretically increasing the possibility of detection developed by ELISA, and do not



**Figure 2.** (a) PCA3 nomogram predicting cancer on prostate biopsy; (b) local regression nonparametric smoothing plots showing the calibration of the PCA3 nomogram; (c) local regression nonparametric smoothing plots showing the calibration of the base nomogram. Instructions for physicians: To obtain nomogram-predicted probability of prostate cancer, locate patient values at each axis. Draw a vertical line to the “Point” axis to determine how many points are attributed for each variable value. Sum the points for all variables. Locate the sum on the “Total Points” line to be able to assess the individual probability of cancer on prostate biopsy on the “Probability of prostate cancer at biopsy” line. Instructions for readers: Perfect predictions correspond to the



458 line. Points estimated below the 458 line correspond to nomogram overprediction, whereas points situated above the 458 line correspond to nomogram underprediction. A nonparametric, smoothed curve indicates the relationship between predicted probability and observed frequency of prostate cancer on initial biopsy. Vertical lines indicate the frequency distribution of predicted probabilities.(62) (Adapted with permission from Elsevier).

require exfoliated cancer cells to be present for detection. A combination of markers may provide improved diagnostic and prognostic accuracy, thus alleviating unnecessary procedures, cost and morbidity.(80)

Three specific proteins as biomarkers for PCa are:  $\alpha$ HGF, IGFBP3 and OPN. These proteins have yet to be identified as urinary biomarkers in PCa, but have been shown to play key roles in PCa initiation and progression. HGF is a pleiotropic cytokine that has been implicated in angiogenesis, adhesion, migration, invasion and proliferation of PCa cells.(81) Elevated quantities of activated HGF have been detected in serum of PCa patients.(82) Similarly, elevated levels of c-Met, the tyrosine kinase receptor for

HGF, have been detected in the urine of PCa patients. (83,84) Current finding shown a significant overexpression of  $\alpha$ HGF in PCa urine samples as compared with controls, supported by evidence of similar findings in serum and plasma HGF biomarker studies.(83,85) In particular, the mechanism of elevating  $\alpha$ HGF can be associated with the pathway involving its receptor, c-met, and proteolytic enzymes, HGFA and matrilysin, which cleave HGF to form a biologically active heterodimer.(82) This suggests that  $\alpha$ HGF levels increase to initiate the cancer phenotype, but  $\alpha$ HGF cannot discriminate between localized and metastatic disease.(86)

IGFBP3, a component of the IGF system, has been reported to be involved with cellular differentiation, survival and proliferation; recent studies have shown higher levels of plasma IGFBP3 to correlate with an increased likelihood of harboring PCa.(87) Urinary  $\alpha$ HGF and IGFBP3 can be used to differentiate between individuals with and without cancer, while OPN levels can be used to identify those with more aggressive disease.

OPN, also known as secreted phosphoprotein 1 (SPP1), is an extracellular matrix protein with a number of diverse roles, including blood vessel formation and tumorigenesis. (88) OPN has been found to have significantly increased expression at the mRNA and protein levels in patients with aggressive PCa.(89) Thalmann *et al.* found levels of urinary OPN to be significantly higher in metastatic samples compared with localized disease and normal samples,

which confirm previous findings that have linked OPN to a malignant phenotype.(90)

### **Tumor Vascularity in PCa**

Tumors require an increased blood supply for growth. Inducing angiogenesis is one of the hallmarks of cancer (91) and a critical mechanism behind tumor dormancy (92). The transition from dormancy to outgrowing vascularized tumor occurs when the balance tips in favor of angiogenesis. This is referred to as the 'angiogenic switch' and is controlled by both anti-angiogenic and pro-angiogenic regulators (93). Neovasculature can arise from the sprouting of new vessels from existing ones (angiogenesis), or *de novo* vessel formation from circulating endothelial precursor cells (vasculogenesis).(91) Tumor vessels are characteristically heterogeneous, in contrast to normal mature blood vessels. (94)

Tumor vascularity in PCa has been linked to disease aggressiveness, where highly vascularized tumors are more responsive.(95) Microvessel density (MVD) has been used as a histological marker of cancer vasculature. MVD can be calculated using analysis of vascular 'hot spots', random area selection, larger representative areas of the specimen or even whole specimen analysis, and automated analysis was used to reduce bias.(96-98) Aggressive prostate tumors are seen to form vessels primitive in morphology and function. Poorly differentiated tumors have greater MVD, irregularity of vessel lumen and smaller vessels. In addition, tumors exhibiting the smallest vessel diameter or the most irregularly shaped vessels have been associated with the development of lethal disease.(99) However, MVD is not consistent across all studies.(96) By contrast, transition zone tumors display a large variability in microvascular parameters. They can be both hypo- or hyper-vascularized compared with normal transition zone tissue.(98) MVD failed to provide an independent prognostic factor when combined with standard predictors in a multivariable analysis.(100) Therefore, MVD has a limited application in the clinical setting.

Circulating endothelial cells (CECs) and circulating endothelial progenitors (CEPs) comprise subsets of cells that are different functionally and phenotypically. Both reflect angiogenesis and have been heralded as promising noninvasive biomarkers for the prediction of prognosis and evaluation of treatment response in cancer. CECs are mature, terminally differentiated cells that are shed from the vessel wall into the circulation in response to injury or as a result of endothelial dysfunction. However, the precise role of CECs in malignancy is unclear. At this stage, CECs are best understood as a product of vascular turnover and, as

such, a surrogate marker of angiogenesis. CEP first isolated in peripheral blood by Asahara and colleagues in 1997.(101) Unlike CECs, CEPs are mobilized from bone marrow (BM). A population of CEPs have clonogenic and proliferative potential (endothelial colony forming cell).(102) CEPs are relatively rare in healthy individuals. Within the vessel wall, BM-derived CEPs are thought to merge and differentiate into endothelial cells.(103)

Many factors contribute to the conflicting body of evidence regarding CEC and CEP levels as biomarkers in cancer. Among these, the most significant is the differences in enumeration methodology. No one marker can uniquely identify CECs and CEPs, and there is no agreement on which combination of markers can identify them reliably, although it is reasonable to hypothesize that these cells have the potential to be valuable biomarkers, given the importance of angiogenesis in cancer. The lack of research in this area probably reflects the methodological challenges of enumeration of these cells rather than a lack of scientific interest.(104)

Platelets play a number of significant roles in metastatic disease.(105-107) One role is the facilitation of certain steps of hematogenous metastasis.(108) Platelets actively signal to tumor cells via the transforming growth factor  $\beta$  (TGF $\beta$ ) and nuclear factor  $\kappa$ B (NF $\kappa$ B) pathways. Inhibition of these pathways protects against lung metastasis *in vivo*. (109) Metastasis can be significantly reduced through depletion of platelets or inhibition of tumor cell induced platelet aggregation.(107) Platelets are also able to protect tumor cells from attack by the immune system, by limiting the ability of natural killer cells to lyse tumor cells *in vitro* and *in vivo*.(107) Further investigation showed there was no difference in total platelet count between patients who did and did not recur (110), but a subset of platelets showed significantly correlated with early biochemical recurrence in PCa after prostatectomy. Although platelets have a well established role in cancer, some large investigation in exploratory studies and further evaluation in prospective trials should be established before recommendations can be made regarding their use in routine PCa care.(104).

### **Circulating Tumor Cells (CTCs)**

As a tumor progresses, it sheds its cells into the bloodstream and these cells may form distant metastases. Detecting and measuring CTCs by isolating them and performing reverse transcriptase polymerase chain reaction (PCR) of PCa - specific genes has shown promise in the diagnosis and prognosis of PCa. Changes in CTC levels may be more accurate than PSA in predicting outcomes for castration-resistant prostate cancer (CRPC).(111) Patients with

a CTC count of more than 5 CTCs/7.5 mL blood have a significantly reduced overall survival compared to patients with less than 5 CTCs/7.5 mL blood. (112,113) PCa CTCs are reported to reflect those mutations present in the primary tumor *e.g.*, TMRSS2-ERG fusions, androgen receptor mutations, and Phosphatase and Tensin Homolog (PTEN) deletion which, together with PSA, alpha-methylacyl-CoA racemase (AMACR) and androgen receptors, can predict the response to treatment.(114,115) The number of CTCs present in whole blood might allow for determination of cancer burden, and provide a more readily accessible source of molecular information of the primary tumor. Despite their promise and proposed function, CTC detection remains a major technical challenge (116) and their clinical relevance remains controversial. In addition, the labor-intensive nature of isolating CTCs, high cost and the extremely low numbers in blood is a technical hurdle, especially in the early stages of PCa.(117)

### TGF $\beta$ -1

TGF- $\beta$ 1 is a ubiquitous growth factor that has been implicated in several molecular processes relating to cell proliferation and differentiation, cytokine response during inflammation and new blood vessel growth. TGF- $\beta$ 1 has been shown to be overexpressed in PCa tissue specimens and correlates with tumor grade and metastasis.(118) TGF- $\beta$ 1 also correlate with prostate tumor extravasation and biochemical recurrence.(119) Furthermore, circulating TGF- $\beta$ 1 has been shown to be elevated in PCa patients. (120) In combination with other markers, TGF- $\beta$ 1 could prove to have clinical utility for PCa prognosis.

### Autoantibodies in PCa

Cancers are known to activate the cellular immune system, including the mounting of an autoimmune response to antigens presented by the tumor.(121) Detection of autoantibodies produced against AMACR in PCa patients in the gray zone of 4–10 ng/ml were shown to stratify PCa from non-PCa with a sensitivity of 62% and specificity of 72%.(122)

### AMACR

Immunohistological markers of PCa are also important in distinguishing between prostate tumor stages during biopsy analysis. AMACR is an enzyme involved in the synthesis and metabolism of fatty acids and has been shown to have high expression in prostate tissues, about 80-100% in PCa tissues (123), detected in blood and urine with a high sensitivity and specificity (122,124,125). AMACR also correlates with PCa metastasis and biochemical recurrence

when levels are lowered, and its inhibitors have potential to provide a novel treatment for CRPC. However, AMACR is also expressed in many other tissues, thus limiting its utility as a tissue marker for PCa.(124)

## Circulating Nucleic Acid as Biomarkers of PCA

Cell-free circulating DNA or mRNA are attractive to clinicians and scientists because of their potential for minimally invasive detection and monitoring of disease pathogenesis, but some technical challenges in terms of sensitivity, specificity and/or nucleic acid stability are still in considerate currently. 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.(126) In prostate cancer, three types of DNA alterations have been investigated as plasma/serum biomarkers. These are mitochondrial DNA (mtDNA) mutations (127-130), microsatellite instability (MI) (131-135), and gene promoter hypermethylation (132,135-145).

### Hypermethylation Event

Hypermethylation of CpG islands within the promoter of the gene encoding GSTP1, a tumor-suppressor protein involved in detoxification processes, has been described as one of the earliest events in prostate carcinogenesis and leads to loss of gene expression.(126) Measurements in urine after prostatic massage have shown that decreased expression of GSTP1 mRNA correlates with positive biopsies.(146,147) In addition, the promoter methylation status of GSTP1 in urine has been measured and shown to have specificities of 93–100% for PCa detection and sensitivities of 21.4–38.9%.(148-151) However, it was shown in other studies that after prostatic massage the sensitivity increased to 75%.(152,153)

### MI

Microsatellites are repeated sequences of DNA made of repeating units of 1-6 base pairs in length. Microsatellite stretches may be disrupted by base substitutions (imperfect microsatellites) or insertions (interrupted microsatellite). MSI structure consists of repeated nucleotides, most often seen as GT/CA repeats. A higher number of repeats causes a higher mutation rates (154).

Increased frequency of MI markers was identified in patients with metastatic prostate cancer.(131) 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.(126)

### mtDNA

Jeronimo *et al.* sequenced the D-loop region, 16S ribosome RNA (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. However, such mutations were a relatively rare event, with mtDNA mutations identified in only three of 16 patients examined, limiting the diagnostic potential of such mutations.(128)

mtDNA appears to be of greater prognostic than diagnostic utility in prostate cancer serum/plasma, particularly in advanced prostate cancer, where patients who did not survive to 2-year follow-up had 2.6-fold higher circulating mtDNA level at initial presentation than surviving patients.(129)

### Circulating mRNA

The utility of circulating mRNAs as biomarkers is hampered by the low specificity of quantitative PCR (qPCR)-based assays, and use of target mRNAs that are prostate-specific, but not always prostate cancer-specific. Circulating mRNA is less stable than circulating DNA, resulting in lower abundance of mRNA targets for qPCR applications. Thus, circulating mRNAs have demonstrated potential for distinguishing patients with organ-confined disease from those with metastatic disease (126).

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 PCa abilities of prostate cancer cells. Plasma BMP6 mRNA levels, in combination with PSA, can be used as an indicator of disease progression and/or treatment response.(155,156)

The lengths of the telomeric ends of chromosomes are maintained by the enzyme human telomerase reverse transcriptase (hTERT). Overactivity of hTERT has been shown to be present in 90% of PCa tissues.(157) 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.(158) 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 anterior gradient 2 (AGR2) levels have been investigated as a putative diagnostic prostate cancer biomarker.(159,160) AGR2 mRNA levels are significantly elevated in patients with metastatic prostate cancer, and are highest in patients with clinicopathological indicators of NP-CRPC (161). AGR2 mRNA levels may be used as an aid to noninvasively identify patients with NP-CRPC and to subsequently assist with treatment planning (161).

### Circulating micro RNA (miRNA)

miRNAs are naturally occurring single-stranded RNA molecules, 19-25 nucleotides in length, PCa able of post-transcriptional regulation of target mRNAs to which they bind, at complementary sequences most frequently in the 3'-untranslated region. 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.(162) 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).(163) Tumor cells release miRNAs into the blood and circulating expression profiles of miRNAs are altered in many tumor types, suggesting that miRNA profile can be informative about the disease.(164,165) Furthermore, detection and quantitation can be relatively easily achieved in low volumes of blood serum or plasma qPCR, which is both specific and sensitive.(166) PCa associated miRNAs in serum allow for minimally invasive diagnostic separation of samples from tumor burdened and healthy patients. miR-21, miR-125b, miR-221 and miR-222 are part of the oncogenic miRNA family that are upregulated in human aggressive PCa.(167) miR-21 is overexpressed in PCa and other tumors acting as an oncogenic regulator leading to tumor growth (168) by silencing PTEN and other tumor suppressing genes.(169) The miR-200 family has recently generated interest in PCa research due to their lowered expression in PCa. A study of a Chinese population (140) identified a panel of five miRNA markers (let7-c, let7e, miR-30c, miR-622 and miR-1285) that differentiated PCa from benign and healthy control samples. However, for a larger clinical utility, these circulating nucleic acid biomarkers require extensive and detailed standardization and confirmation.

## Proteomic, Genomic and Metabolomic Approaches to PCa Biomarker Discovery

The emergence of the 'omics' era has created great insight into the mechanisms and networks involved in disease progression and etiology. Specifically, proteomics has provided information on the post-translational fate of genes, through the analysis of protein expression levels and post-translational modifications. A challenge with proteomic analysis of biological fluids such as plasma and serum is the large dynamic range of protein concentrations. (171) Increasing improvements in genomic technologies facilitated the migration from array-based methods to 'next-generation' sequencing platforms. Metabolomic analysis of PCa tissues and urine identified that sarcosine tissue levels correlate with PCa progression and metastasis.(172)

## Conclusion

Early diagnosis and accurate prognosis of organ-confined PCa coupled with identification of predictive markers that can be identified to guide treatment options is still the goal that the PCa research community is striving towards. The discovery of novel noninvasive markers would aid in this effort tremendously by reducing biopsy procedures, surgeries and treatments for men who would not see a benefit. Currently, no single test can achieve the above goals and we predict that one single biomarker will not be able to fulfill the above requirements for the next PCa screening tool. Due to the heterogeneity of the disease, no one biomarker will be diagnostic and prognostic for every patient. On this basis, we summarize that the next biomarkers for PCa will most likely be an assay employing multiple biomarkers assayed in combination using protein and gene microarrays, containing markers that are differentially expressed in PCa.

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