[Frontiers in Bioscience E2, 641-656, January 1, 2010]

Molecular markers for prostatic cancer

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1. ABSTRACT

Prostate cancer (caP) is a major public health problem. Many groups have attempted to identify prognostic risk factors to early detect caP and to identify who will need active treatment. Since the introduction of prostate specific antigen (PSA), diagnosis of caP has increased even as mortality for prostatic cancer has declined. Using current recommended guidelines, the PSA test suffers from both of limited specificity and sensitivity. With the aim to improve early detection of prostatic cancer the volume adjusted PSA, PSA isoforms and PSA kinetics have been investigated. Recently, technological advances in molecular assays have led to the discovery of new markers with high specificity. Further, proteomic array profiling and DNA methylation assays could provide for more accurate diagnosis and prognosis. Current evidence suggests that no single marker is likely to achieve the desired level of diagnostic and prognostic accuracy: future research should focus on validation of already existing biomarkers and the discovery of new markers to identify men with aggressive prostate cancer and to predict outcomes after therapies.

2. INTRODUCTION

Prostate cancer is the most common solid neoplasm among European men, with an incidence of 214 per 1000, out numbering lung and colorectal cancer (1). Many groups have attempted to identify prognostic risk factors based on clinical, serological and pathologic parameters to early detect prostate cancer and to identify who will need active treatment (2, 3).

Since the introduction of prostate-specific antigen (PSA) testing in the late 1980s, prostate cancer diagnoses have increased, even as mortality rates for prostate cancer have declined. Over the past few years, there has been increasing recognition that not all men diagnosed with prostate cancer require treatment. Indeed, the 5-year survival for prostate cancer is over 98%. The landscape for management of the disease has further changed with the recognition that many men diagnosed with low-risk prostate cancer (organ-confined, Gleason 6 prostate cancer) will not require definitive therapy for their cancer due to the low risk of morbidity and

mortality. Within the past decade, advances in proteomics have stimulated a search for new biochemical markers with increased specificity.

Biochemical markers in oncology are molecules that can be detected in higher or lower than normal amounts in the blood, urine, or body tissues of some people with certain types of cancer. A tumour marker may be produced by the tumour itself, by the surrounding normal tissue in response to the presence of tumour or by repetitive lesions.

They are different types of molecular tumour markers including DNA, mRNA, proteins, antigens, or hormones measured quantitatively and/or qualitatively by appropriate assays. Tumour marker assays comprise immunohistochemical (IHC) test, quantitative immunoassays, polymerase chain reaction (PCR), western or northern blot and more recently micro arrays (genomic and proteomic) and mass spectrometry.

Tumour markers could identify a disease process, a specific tissue or patient's characteristics and help establishing the severity and extend of the disease. They are usually not used alone for the diagnosis because most markers can be found in elevated levels in people who have benign conditions, and because no tumour marker is yet specific to a particular cancer. Not every tumour will cause an elevation in the tumour marker test, especially in the early stages of cancer.

With these limitations tumour markers may be however useful for the four following clinical purposes: a) screening a healthy population for the presence of cancer or for detecting a group at a higher risk for developing a cancer; b) making a diagnosis of cancer: a diagnostic tumour marker is a marker that will aid in detection of malignant disease in an individual. Preferably, the marker should be tissue specific and not influenced by benign diseases; c) determining the prognosis in a patient with cancer. This would provide to the clinician a tool for early prediction of tumour recurrence, progression and development of metastases, following the initial surgical removal of the cancer but without administration of adjuvant therapy; d) monitoring efficacy of anti-tumoral treatment: tumour markers may predict how the patient is going to respond to a given therapy which includes surgery, radiation, chemotherapy or more recently targeted treatments.

Due to the complexity of the pathophysiology of prostatic cancer development, a large number of molecular tumour markers have been suggested. (Table 1). We review the literature for prostatic cancer serum and urine molecular markers and they will now be discussed.

3. PROSTATE SPECIFIC ANTIGENS (PSA), PSA ISOFORMS AND PSA DERIVATE

Prostate specific antigen (PSA) is serine protease produced at high concentrations by normal and malignant prostatic epithelium. Before the advent of PSA as a biomarker for prostate cancer, practitioners relied on the use of DRE, PAP and TRUS for the screening, diagnosis and staging of cancer of the prostate.

With DRE as the primary detection method, cancer detection rates have been previously estimated to be about 1% to 2% in self-referred screening populations and between 48% and 85% of cancer detected with DRE are, at diagnosis extraprostatic, non-organ confined. The discovery of PSA and its integration into urological practice with DRE and TRUS provided the opportunity to detect prostate cancer during the window of curability, improved the assessment of disease extent after diagnosis, and provided a method for monitoring the success of prostate cancer treatments. PSA was first identified in seminal plasma in 1966 and called gamma-seminoprotein (gamma-SM). Clinically important as a forensic marker, the seminal specificity, stability (identifiable for as long as 1 year), and ease of identification of PSA made it an ideal marker for identification of semen in cases of sexual assault. PSA was first described in plasma by Wang *et al* in 1979 (4).

Rabbit antiserum was raised against a crude extract of prostate tissue. This antiserum was able to detect, via gel electrophoresis, a protein antigen that was separate from PAP, present in normal prostate, BPH, and prostate cancer tissue exclusively. A pure form of the protein was obtained through column purification and gel electrophoresis, and later in the 1990s it was confirmed that previously identified proteins in seminal plasma (gamma-SM, p30) were identical to the protein found by Wang *et al* (4).

PSA belongs to the human kallikrein (hK) family and is also known as hK3; its function is to digest the gel that is formed in semen after ejaculation (5, 6, 7). Normally, only a minor fraction of PSA leaks into the extracellular space and into circulation. When tissue architecture is distorted in Prostatic cancer, serum PSA will increase. PSA is produced as a pre-proenzyme comprising 261 amino acids, including a signal peptide that is 17 amino acids long and is removed during synthesis. The secreted proenzyme (proPSA) thus contains 244 amino acids including a 7 amino acid activation peptide, which is split off after secretion. Mature (free) PSA thus contains 237 aminoacids. PSA isolated from seminal fluid is partially degraded by proteolytic cleavage (i.e. nicking) at certain sites, producing nicked free PSA. PSA detected by immunoassays is mainly complexed (cPSA) with protease inhibitors, predominantly with a1-antichymotrypsin (ACT), while minor parts occur in complex with a2-macroglobulin (AMG) and a1-protease inhibitor (API). A certain percentage of PSA (i.e. 5–35%) will remain unbound (8).

Serum PSA is the most widely used biomarker for the screening and early detection of prostate cancer. Higher PSA levels are directly associated with the risk of cancer and the risk of high grade disease as well as with tumor stage (9). There is a non negligible risk of prostate cancer at any PSA level, making it difficult to recommend

Table 1. Molecular markers for prostatic cancer detection

Marker	Specimen	Description	Biological Function	Purpose	Detection methods
PSA	Serum	serine protease, family of human kallikrein(Hk3)	digest the gel that is formed in semen after ejaculation	Diagnosis, Prognosis, Follow up	Commercially avaible serum immunoassays
Human kallikrein 2	Serum	Serine protease with trypsin-like substrate specificity	Cleaves pro-PSA to form PSA	Diagnosis, Prognosis, Follow up	Research and commercially avaible
GSTP-1[1]	Tissues Urine Seminal fluid/expressed prostatic secretions	CpG[2] island hypermethylation of DNA encoding the protein, glutathione S-transferase	Hypermethylation silences transcription; GSTP1 normally inactivates oxidant and electrophilic carcinogens via conjugation to reduced glutathione	Diagnosis	RT-PCR[3], methylation-specific PCR assay, and PCR-restriction fragment length polymorphism
TMPRSS2:ERG[4] **fusion products**	Urine	androgen responsive membrane anchored serine protease (TMPRSS2)	Unknown	Diagnosis, Target terapy	RNA amplification and quantitative PCR
PCA3[5]	Tissue Urine	Prostate specific non-coding RNA	Unknown	Diagnosis	Commercially avaible Transcription mediated amplification technology to quantify PCA3 and PSA mRNA in urine samples collected after a DRE[6]
AMACR[7]	Serum Tissue urine Seminal fluid/expressed prostatic secretions	382 amino acid (approximately 44 kDa) peroxisomal and mitochondrial enzyme up regulated in PCA	Bile acid synthesis, peroxisomal β-oxidation of branched-chain fatty acids and conversion of branched-chain fatty acids from R-stereo-isomers to S-stereo-isomers	Diagnosis	RT-PCR[3] immunoblot analysis and ELISA[8]
PSMA[9]	Blood/serum	Cell surface peptidase,	involved in hydrolyzing peptides in prostatic fluids, signal transduction, cell migration, and nutrient uptake. Also potential receptor function	Diagnosis, Target terapy	RT-PCR[3]
EPCA[10]	Serum	Prostatic cancer associated nuclear structural protein	Possibly involved in early prostate carcinogenesis	Diagnosis Prognosis	ELISA[8]
Chromogranin A	Serum	Pro-hormone peptide released by neuroendocrine cells	Unknown	Prognosis	Detectable using a quantitative sandwich immunoassay, immunoradiometric assay, or ELISA[8]
Neuron Specific Enolase	Serum	Enzyme found in mature neurons and cells of neuronal origin.	phosphopyruvate hydratase	Prognosis	ELISA[8]
Gastrin-releasing peptide	Serum	27 amino acids peptide	regulatory human peptide that elicits gastrin release and regulates gastric acid secretion and motor function; involved also in epithelial cell proliferation	Diagnosis Prognosis	ELISA[8]

Abbreviation: [1]glutathione S-transferase p1; [2]cytosine guanine; [3]reverse transcriptase-polymerase chain reaction; [4]transmembrane protease, serine 2; [5] prostate cancer antigen 3; [6]digital rectal examination; [7]alpha-methylacyl-CoA racemase; [8]enzyme-Linked ImmunoSorbent Assay; [9]prostate-specific membrane antigen; [10] early prostate cancer antigen.

lower PSA cut-off for a recommendation for more invasive screening (10).

Multiple large sized population-based studies show unequivocal evidence that also only a modest elevation of the blood level of PSA above population-based averages is strongly associated with increased cancer risk (11, 12, 13, 14, 15). However, it is also widely documentated that the frequency of BPH increases sharply above age 50 and that it also causes the PSA levels to rise in the blood. This helps to explain why PSA elevation may be common to both benign and malignant prostate disease, and why PSA is not a cancer, albeit-tissue specific biomarker.

Indeed, as a group, in men with PSA below 4.0 ng/ml the risk of cancer is approximately 15% and 15% of these patients have high grade disease (16).

Nonetheless, at lower PSA levels, for example less than 1.0 ng/ml, the risk of high grade disease is quite low. Conversely while PSA levels above 4.0 ng/ml have traditionally been deemed increased, cancer is found on biopsy in only 25% to 30% of the men evaluated. The operating characteristics of all other cut-offs for PSA, in addition to 4.0 ng/ml, are similarly challenging from a clinical standpoint when tradeoffs in sensitivity and specificity are examined (10).

To improve its operating characteristics several modifications of PSA have been introduced, including the rate of change in PSA with time (PSA velocity), the ratio of PSA to prostate volume (PSA density), age specific PSA ranges and PSA doubling times (17, 18, 19, 20).

PSA velocity exceeding 0,75ng/ml/yr was associated with higher risk of prostatic cancer than a slower rise in PSA over time (21). Recent evidence suggests that

this cut-off point is useful only for men with PSA more than4ng/ml. In younger men with a lower PSA level, PSA-V cut-off values of 0.3-0.5 ng/ml/year were suggested as a basis to perform a biopsy (22). This study demonstrated that PSA-V may improve the predictive ability of a model incorporating PSA; however, results must be interpreted with caution due to verification bias in the study design. In a study from the PCPT, investigators demonstrated that PSA-V within 3 years of diagnosis was predictive of PCA diagnosis (23). However, when added to a predictive model that included PSA level, PSA velocity did not add independent value in predicting cancer risk. Verification bias is not an issue in this study. Further weakness of serial PSA measurements is related to significant inter-assay variations and to significant physiologic between-day (biologic) variation of PSA levels, which has significant implications for screening and diagnosis (24, 25, 26).

D'Amico *et al* investigated whether pre-treatment PSA-V could predict tumor stage, grade, and time to biochemical recurrence (BCR) after RP. This study reported significantly shorter time to PSA relapse and death from prostatic cancer in patients with an annual PSAV of more than2.0 ng/ml/yr among 1054 patients in the year prior to diagnosis (27). The outcome prediction of an elevated preoperative PSA-V has been validated in other surgical series (28). However, PSA-V has not been demonstrated to be an independent prognostic predictor of outcome after therapy when added to a model that includes PSA alone.

The PSAD is defined as the quotient of the serum PSA level divided by the volume of the prostate gland. This calculation takes into account the concept that serum PSA levels increase proportionally with the volume of the prostatic epithelium. Initially, several studies showed that men with prostate cancer had significantly higher PSAD values, typically more than0.10 or 0.15, than men with BPH, thereby improving the specificity of PSA (29, 30).

Unfortunately, several other studies were unable to replicate these results (31, 32).

Because the major determinant of the serum PSA level in men with no prostate cancer is the volume of the TZ epithelium and not the peripheral zone epithelium, the PSAD-TZ was considered as a better alternative to PSAD.

The potential usefulness of this alternative was bolstered because the TZ is enlarged in men with BPH and it is a relatively infrequent site of adenocarcinoma. Unfortunately, while some investigators showed that adjusting for the TZ volume enhanced the diagnostic specificity of PSA, others could not confirm these findings (33, 34). Moreover, given the cost and invasiveness associated with TRUS of the prostate, and concerns about the reproducibility of volume measurements, the PSAD and PSAD-TZ are not used routinely in evaluating men for the presence of cancer or BPH. However, they might be useful in men being evaluated for a possible repeat biopsy.

Age-specific PSA reference ranges are based on the concept that the serum PSA level normally increases as men age. Therefore, age-adjusted PSA thresholds might improve cancer detection in younger men (increase sensitivity) and decrease negative biopsies or minimize the detection of possibly insignificant tumours in older men (increase specificity). Using this approach, Oesterling *et al.* found that a healthy man younger than 50 years should have a serum PSA level less than 2.5 ng/mL, while a man in his seventies should have a level less than 6.5 ng/mL (35). However, Bassler *et al.* reported a significant loss in sensitivity if the upper limit of normal PSA was increased to 4.5 ng/mL in men aged 60–69 years (36). Moreover, when Etzioni *et al.* compared the diagnostic accuracy of PSA at one threshold of 4.0 ng/mL with that obtained at age-specific PSA thresholds, the cancer detection rate was significantly less using age-specific values, even though the positive predictive value was higher (37). In addition, using age-based actuarial estimates of life-expectancy in the USA, and mathematical modelling for life-years gained by the early detection of prostate cancer, Etzioni *et al.* found that the estimated age-adjusted life-years gained by early detection were lower than if the cancer had not been detected, despite the higher potential for detecting younger men with cancer. Thus, the diagnostic utility of age-specific PSA reference ranges remains controversial.

However, none of these modifications have shown operating characteristics that are markedly superior to those of PSA (16-38). These modified biomarkers tend to correlate highly with PSA and the few studies that appropriately evaluated their independent diagnostic contribution to PSA, by simultaneously including PSA and the proposed derivative in the same risk model, showed no incremental value above PSA (38).

Since these PSA derivatives are more difficult to measure than PSA, for example PSA density requires transrectal ultrasound, it is unlikely that they will replace PSA for prostate cancer screening.

New PSA assays have also been developed, including percent free (unbound) PSA, percent complexed PSA and PSA isoforms. PSA in serum may be free or complexed: the amount of unbound PSA, expressed as the free-to-total PSA ratio (percent free PSA), has been used to improve the operating characteristics of PSA, especially in patients with PSA values in the uncertain range between 4 and 10 ng/ml. Free PSA levels below 15% to 25% are associated with an increased risk of prostate cancer but it is estimated that only 30% to 50% of men with free PSA less than 15% have a positive biopsy (39). Complexed PSA was shown to moderately improve specificity by 6.2% to 7.9% compared to total PSA in the PSA range 2.0 to 10.0 ng/ml in a prospectively performed multicenter clinical trial but the AUC for complexed PSA over all PSA ranges only exceeded that from PSA by 1.5% in the same trial (40). This value was quite similar to the approximately 70% reported for PSA in PCPT (38).

Free PSA comprises at least 3 inactive isoforms, including proPSA, BPSA and an additional form of "intact

PSA": assays for these isoforms have been proposed for enhancing PSA accuracy. ProPSA is an initially produced inactive PSA form that includes a seven-amino acid leader peptide sequence. Human glandular kallikrein 2 (hK2) activate this pro-PSA form by removing this seven-amino acid leader peptide sequence. Compared with BPH-associated TZ epithelium, cancer tissues contain higher levels of truncated forms of pro-PSA with either 2 (−2pro-PSA) or 4 (−4pro-PSA) unclipped amino acids from its leader sequence (41).

Pro- PSA improves the detection of prostate cancer in PSA ranges less than 4 ng/ml and it is more highly associated with aggressive prostate cancers than other PSA forms, such as PSA-alpha1 antichymotrypsin and free PSA (42, 43, 44).

BPSA represents benign tissue and has been associated with TZ hyperplasia, which occurs in men with BPH (45). In healthy men, BPSA levels are almost undetectable. The median serum BPSA level in patients with symptomatic BPH is significantly higher than that in patients with no symptoms of BPH (41). BPSA correlates better with TZ volume than does total PSA, and can predict clinically significant prostate enlargement better than total PSA or %free PSA (46). Furthermore, the relation of BPSA and %free PSA to total prostate and TZ volumes is independent of age. Currently, BPSA appears to be promising as a specific serum marker for BPH but not prostate cancer.

The ratio between proPSA and BPSA was proposed to improve the operating characteristics of pro-PSA. Pro PSA/BPSA cut off achieves 90% sensitivity and 46% specificity if fPSA percent is less than 15%.

The third form of free PSA, the 'intact free PSA', is similar to native PSA except that it is enzymatically inactive. Using a newly developed assay that measures intact PSA and pro-PSA, but not BPSA, was found no difference in the absolute levels of this marker between men with and without cancer, but the ratio of this marker to free PSA was significantly higher in patients with cancer (47)

4. HUMAN GLANDULAR KALLIKREIN 2 (Hk2)

Human kallikrein (hK2) is a prostate-specific serine protease that is 80% homologous to PSA. However, while PSA displays chymotrypsin-like specificity, hK2 is trypsin-like. Thus, hK2 can cleave proPSA to form PSA mature (free) PSA suggesting a physiological role of hK2 in the regulation of PSA (48, 49).

PSA and hK2 are both under androgen control but serum levels of hK2 do not parallel those of PSA (serum levels are only 1% of the concentration of PSA): this indicates that it is differentially regulated and suggests that it may be a valuable diagnostic and prognostic marker (50, 51, 52, 53, 54, 55).

Detection at low levels is therefore challenging, but the distinction is perhaps helpful in discriminating normal from cancerous tissue in which serum hK2 levels are more highly expressed (56, 57). Data from Becker reveal that levels of hK2 are undetectable in healthy male controls, whereas concentrations are significantly higher in men with localized prostatic cancer than in men with BPH (58). When used in various combinations with free and total PSA, serum hK2 measurements have significantly improved discrimination of men with and without prostate cancer by increasing both sensitivity and specificity (59, 60, 61). Roobol *et al.* assessed enhancement in predicting biopsy outcome contributed by hK2 to a base model incorporating digital rectal examination (DRE), transrectal ultrasound (TRUS), TRUS-measured gland volume, age, total PSA and having a previous biopsy. Data from this randomized PSA-based screening study of 559 men revealed that hK2 was a significant predictor of biopsy outcome (62).

Several more recent studies have also suggested a role for hK2 in predicting poorly differentiated, locally advanced disease and risk of biochemical recurrence before radical prostatectomy (63, 64, 65, 66). HK2 may be useful in providing better risk assessment and indicating the need for more aggressive therapy.

5. DNA BIOMARKERS

5.1. Epigenetic markers

Hypermethylation of cytosine guanine (CpG) dinucleotide islands at gene promoter regions of tumor suppressor genes has been recognized for a number of tumors as an important event in tumorogenesis, including prostate cancer. CpG hypermethylation is considered to be an initial step in prostate cancer development. A number of different candidate genes have been evaluated, and the most consistently hypermethylated in prostate cancer patients is the glutathione S-transferase p1 (GSTP-1) gene (67, 68, 69).The GSTP-1 gene belongs to a family of enzymes with a primary role in protecting DNA from free radical damage. Loss of GSTP-1 expression due to promoter hypermethylation is the most frequent somatic genome alteration reported in prostate cancer and in high grade prostate intraepithelial neoplasia (70). GSTP-1 may prove to be a valuable biomarker since it is highly prostate cancer specific. It can be detected in prostate cancer tissues, urine and seminal fluid/expressed prostatic secretions. However, significant improvements must be made to improve detection rates in urine. (69, 71) Suggestions for overcoming this problem by performing prostatic massage before voiding have met with mixed results (72, 73).Other candidate genes have been examined for hypermethylation along with GSTP1. Two recent studies looked at a panel of 10 candidate genes (APC, DAPK, ECDH1, GSTP1, MGMT, p14 (ARF), p16, RARbeta2, RASSF1a, and TIMP3) (74, 75).The first study compared urine sediment from 52 prostate cancer patients undergoing radical prostatectomy with that of 91 age-matched controls (74). All 52 prostate cancer patients had at least 1 hypermethylated gene, and 80% had 3 or more hypermethylated genes. The 4 most common genes involved were GSTP1, p16, ARF, and MGMT. All 52 prostate cancer patients had at least 1 of these genes hypermethylated, and none of the 91 controls had

hypermethylation of any of these genes. In the second, more recent study, 95 patients undergoing radical prostatectomy and 38 age-matched controls who had negative prostate biopsies submitted urine after prostatic massage. Eight of the loci had increased methylation in cancer patients over the controls (*P* < .05). The 4 genes with the greatest difference (GSTP1, APC, RASSF1a, and RARbeta2) had sensitivity for prostate cancer detection of 86% and a diagnostic accuracy of 89% (75).

5.2. Gene fusion/translocation markers

Gene rearrangements are associated with a number of cancers, especially leukemias and lymphomas, and recent studies have uncovered gene rearrangements in patients with prostate cancer as well. A recent study identified a gene fusion (translocation) present in 80% of prostatic cancers (23 of 29 prostate cancer tissue samples), in 20% of prostatic intraepithelial neoplasia and absent from benign prostate tissue (76). Among the top 10 over expressed genes found were ERG and ETV1, which were fused to the 5=untranslated regions of TMPRSS2. TMPRSS2 is an androgen responsive membrane anchored serine protease. In a study of 252 men with stage T1a/b prostate cancer followed for a median of 9 years, TMPRSS2: ERG gene fusion was more commonly associated with Gleason scores more than 7 (41% vs. 12%; *P* = .01) and more prostate cancer deaths and/or metastatic disease development (53% vs. 23%; *P* = .03) (77). In univariate analysis, the cumulative incidence ratio was 2.7 (95% CI, 1.3–5.8; *P* < .01) for the association between the TMPRSS2: ERG gene fusion and prostate cancer specific death and/or metastatic disease. After controlling for Gleason score, however, this cumulative incidence ratio did not reach statistical significance (CIR = 1.8; 95% CI, 0.6–5.3; *P* = .2). A urinary test for the detection of TMPRSS2: ERG fusion products has been developed using RNA amplification and quantitative PCR (78). In the pilot study of 19 patients with prostate cancer, urine was collected after prostatic massage. Forty-two percent of the patients had the TMPRSS2:ERG gene fusion detected, consistent with what is found in the tissue analysis data. The researchers confirmed with fluorescence *in situ* hybridization (FISH) analysis on the radical prostatectomy specimens the presence of the TMPRSS2: ERG gene fusion in a subset of the patients. Further work is required, and the assay used was only directed toward 1 of the TMPRSS2: ERG gene fusion isoforms, although this isoform is the most commonly detected (80%–95%) in patients with TMPRSS2: ERG gene fusion. Perhaps these fusion genes ultimately will serve more as targets for therapy than as biomarkers.

6. RNA BIOMARKERS

6.1. PCA3

In 1999, Bussermakers *et al.* reported that PCA3, also referred to as PCA3 DD3 or DD3 PCA3, was over expressed in prostate cancer tissue (79). Since that time, several assays have been developed to measure PCA3 (DD3 messanger ribonucleic acid) levels, in urine specimens typically collected after digital rectal examination. In 2007, Marks *et al* evaluated PCA3 in 233 men with PSA level more than2.5 ng/ml and at least one prior negative prostate biopsy (80). They showed that urine PCA3 levels were more accurate than serum PSA measurements for predicting the results of repeat biopsy (area under curve (AUC):0.68 vs. 0.52, p=0.008).The Progensa TM PCA3 test (Gen-Probe, San Diego, CA, USA) uses transcription mediated amplification technology to quantify PCA3 and PSA mRNA in urine samples collected after a DRE. The DRE is required to release prostate cells into the urine and the quantification of PSA mRNA is required to normalize for the total mRNA present in a sample (PSA mRNA levels in prostate cells released into urine are completely unrelated to PSA protein levels in blood and are essentially unchanged in prostate cancer (81). Thus, the method measures both PCA3 mRNA and PSA mRNA, and the results are represented as a ratio of the two mRNAs, referred to as the 'PCA3 score'. Similar to other gene-based tests, the PCA3 assay is comparable in cost and complexity. Samples must be sent to a qualified laboratory experienced in performing molecular testing and PCA3 scores are reported to the urologist. Informative rates (percentage of urine samples yielding accurately quantifiable mRNAs for assay) are more than 99% (82), and the assays have good reproducibility with intra- and inter-assay coefficients of variation of <13% and <12%, respectively, and total variation of< 20% for the PCA3 score (83).

Determining a PCA3 score could be useful in several clinical scenarios. First, the score can be used to increase confidence in an initial biopsy decision where the serum total PSA results are uncertain (2.5–10 ng/mL). Second, PCA3 testing could be used to increase confidence in a re-biopsy decision, wherein the DRE and serum total PSA results are suspicious and/or family history and other factors indicate an increased risk of prostate cancer. Lastly, when biopsy results are positive but tumour aggressiveness is unknown, PCA3 might be useful in comparing the risks and benefits of radical prostatectomy vs. active surveillance management. Thus, the availability of a PCA3 score alone or combined with existing methods might better guide biopsy decision making than current methods, and might be useful as an indicator of clinical stage and disease significance. Comparative research studies have consistently shown a better predictive value for prostate cancer for PCA3 than for serum total PSA. In a separate study, Groskopf compared PCA3 and total PSA in 70 men who had a prostate biopsy based on pre-existing risk factors, in comparison with 52 apparently healthy men with no known risk factors (84). At a PCA3 score threshold of 50, the sensitivity was 69% and the specificity 79%. For serum total PSA at the established threshold of 2.5 ng/mL, and with sensitivity held constant at 69%, the specificity for total PSA was 60%.The foregoing results were recently confirmed using a time-resolved fluorescence-based variant of the PCA3 test by van Gils *et al.* In their multicentre study of 583 men with a serum total PSA level of 3–15 ng/mL, the AUC for predicting a positive biopsy was higher for PCA3 than for serum total PSA testing. There was also a correlation of increasing PCA3 score with increasing probability of positive repeat biopsy (85). Although showing great possibilities, PCA3 still requires

additional work. The literature on PCA3 uses a number of different assays and thresholds for prostate-cancer detection, raising questions of reproducibility and standardization.

6.2. Alpha-methylacyl CoA racemase

One such potential marker is alpha-methylacyl-CoA racemase (AMACR), an enzyme that is involved in peroxisomal beta-oxidation of dietary branched-chain fatty acids. Recent studies have shown that, compared with expression in normal or benign prostate epithelium, AMACR is consistently overexpressed in prostate cancer epithelium, making it a specific marker for cancer cells within the prostate gland. Furthermore, over expression of AMACR may increase the risk of prostate cancer because its expression is increased in premalignant lesions (prostatic intraepithelial neoplasia) (86, 87, 88). In addition, epidemiologic, genetic, and laboratory studies point to the importance of AMACR in prostate cancer. Indeed, there is an association between higher dietary intake of the main sources (e.g., beef) of branched-chain fatty acids and prostate cancer risk; genome-wide scans for linkage in hereditary prostate cancer families suggest that the chromosomal region for AMACR (5p13) is the location of a prostate cancer susceptibility gene, and AMACR gene sequence variants (polymorphisms) co-segregate with cancer of the prostate in families with hereditary prostate cancer (89, 90, 91, 92). Also, experimentally induced loss of AMACR expression slows the growth of some prostate cancer cell lines (93). Thus, because AMACR has been shown to be detectable in the urine of men with prostate cancer after prostatic biopsy, AMACR may be a valuable biomarker for prostate cancer (94). Sreekumar *et al.* screened sera from men with biopsy-proven prostate cancer and men without known prostate cancer for a humoral immune response to AMACR, because it has not been possible to consistently detect AMACR in the circulation. Using protein microarrays, they found that AMACR immunoreactivity was statistically significantly higher in the sera from cancer case subjects than from control subjects; this finding was not seen for other proteins on the microarray. Interestingly, all men showed evidence of a humoral response to PSA, regardless of cancer status (95).

Specificity of the AMACR immune response was validated by using quantitative immunoblot analysis and an enzyme-linked immunosorbent assay, with the results of both approaches suggesting the presence of high-affinity antibodies to AMACR in the sera of prostate cancer case subjects. In addition, the immune response against AMACR (when used to discriminate between cancer patients and control subjects) had a statistically significantly greater sensitivity and specificity than that of the PSA test.

AMACR is consistently over-expressed in prostate cancer with high specificity (79%–100%) and sensitivity (82%–100%) (96). In fact, antibodies against AMACR are commonly used for immunohistochemistry analysis of prostate biopsies to help distinguish benign from malignant tissue. Elevated levels of AMACR mRNA have been detected in prostate cancer patients by RT-PCR from serum, urine, and prostatic secretions although the work has been limited to small series and primarily limited to proof of principle (97, 98).

Circulating levels of AMACR protein are quite low, making development of a serum test difficult, but some investigators have demonstrated AMACR protein levels in urine by western blot analysis. (95) Additionally, one group was able to detect higher levels of antibodies to AMACR in patients with prostate cancer compared with those without cancer with a sensitivity and specificity of 62% and 72%, respectively (95). Further studies of AMACR are underway to determine its clinical applicability.

7. PROSTATE ANTIGENS

7.1. Prostate-specific membrane antigen

Since its discovery in 1987, prostate-specific membrane antigen (PSMA) has been evaluated extensively as a diagnostic and prognostic marker although little is known of its functioning role. Despite significant expression in prostatic cells, detecting circulating PSMA by western blot or enzyme-linked immunosorbent assay (ELISA) is imprecise for quantitating proteins, and studies using these techniques have revealed mixed results. Initial reports indicated prognostic value of PSMA serum levels in select patients but further data showing additional value to current predictors have yet to be reproduced (99,100,101,102).

In an effort to improve clinical staging, reverse transcriptase (RT)-PCR has been applied to detect PSMA-producing cells in the circulation (103). However, too few institutions report a positive correlation for validation, and a current prospective study revealed no association between PSMA RT-PCR prevalence and pathological stage or biochemical relapse after radical prostatectomy (104, 105, 106). In an attempt to better quantify circulating levels of PSMA, Xiao *et al* have employed a novel protein biochip immunoassay. Their initial results show that levels of serum PSMA in caP patients are significantly different from those with BPH and in healthy men, even after stratification by age (107). Further testing is needed to validate the application of this assay.

Combined assays of more than one RT-PCR marker (that is, PSMA and PSA) have also been examined. Improvement in sensitivity and prediction of extracapsular extension has been reported by several authors, suggesting an advantage in detecting caP micrometastases during clinical monitoring, but the clinical utility of combining tests remains questionable (102, 103, 105).

The use of PSMA as serum marker has limitations, since elevated levels have been observed in healthy men and with increasing age, a potential confounding factor within the population most frequently diagnosed with caP (108). To date, PSMA in tissue has been more encouraging as a diagnostic marker and therapeutic target than a circulating biomarker; however, as new antibodies to PSMA and improved assays are assessed, the value and efficacy of this tumor marker in circulation will be determined.

7.2. Early prostate cancer antigens

Early prostate cancer antigens (EPCA) are prostate cancer-associated nuclear structural proteins, originally identified through protein profiling of rat prostate tissue (109). Immunostaining using antibodies against EPCA peptides in prostate biopsy specimens revealed sensitivities and specificities of 80–100% for detecting prostate cancer (110,111). Positive staining for EPCA was also reported in the precursor lesions proliferative inflammatory atrophy and prostatic intraepithelial neoplasia, suggesting that EPCA might be related to an early step in carcinogenesis. Another interesting finding was that EPCA expression was detected not only in tumour samples, but also in noncancerous tissues adjacent to tumour.

A blood test using an EPCA ELISA was able to identify patients with prostate cancer among plasma samples of 12 patients with prostate cancer and 34 with other diseases, with 92% sensitivity and 94% specificity (112). In another recent study, the EPCA-2 serum ELISA assay had 92% specificity (95% CI, 85–96%) and 94% sensitivity (93–99%) for detecting prostate cancer, using a threshold value of 30 ng/mL (113). In men with a diagnosis of prostate cancer, EPCA had a sensitivity of 90% and 98% in men with organ-confined and extraprostatic disease, respectively.

Interestingly, the EPCA-2 serum assay was effective in differentiating patients with localized disease from those with extraprostatic extension, with an AUC of 0.89 ($P< 0.001$) (113). While larger trials need to be done, these promising results suggest that serum assays for EPCA proteins could be a powerful adjunct to PSA for the early diagnosis of prostate cancer.

8. PROTEOMICS

Alterations in the composition of the proteins in serum could reflect the biology of various tissues and therefore could be used to distinguish patients who have malignant conditions from those with benign disease. potentially prognostic markers for metastatic prostate cancer patients hormonally treated (122, 123).

9.1. Chromogranin A (CGA)

It is known that serum CGA is elevated in various types of endocrine neoplasms (pheochromocytoma, pancreatic islet cell tumors, carcinoid tumors and medullar carcinoma of the thyroid), even if other diseases or drugs (renal impairment, proton pump inhibitors), could influence CGA levels. In recent years CGA has been recognized as a useful marker of these tumors (124, 125, 126, 127).

Angelsen *et al* noted that 91% of prostate glands had neuroendocrine (NE) cells and Prostate cancer patients with CGA-positive tumor cells had elevated serum CGA, although immunohistochemical findings and serum levels of other NE markers, such as NSE, chromogranin B, thyroid stimulating hormone and pancreastatin, did not correlate, suggesting that CGA should be a useful marker for predicting the extent of neuroendocrine differentiation

Promises arising from the initial sets of data (28) were later strongly challenged due to concerns for important biases regarding lack of standardization in the collection and processing of samples, analytical protocols, but also the interpretation after the analytical process (114, 115, 116, 117). Recently was demonstrated that a series of informative peptides present in serum processed according to a highly standardized protocol could efficiently discriminate between three different types of metastatic cancer in samples from patients with either prostate ($n = 32$), bladder ($n = 20$), or breast ($n = 21$) cancer, and discriminate from the proteomic profiles found in healthy volunteer controls without cancer ($n = 33$). This was then validated with an external group of patients with caP ($n = 41$). Sixty-one signature peptides fell into several tight clusters thereby conferring cancer type-specific differences (118). Further research may refine these signature proteins to identify a surrogate marker for detection and classification of disease. However, a recent multi-institutional consortium report was unable to discriminate men with caP ($n = 181$) from men with BPH ($n = 143$) or healthy controls ($n = 220$) using surface-enhanced laser desorption/ionization-based serum proteomic profiling (119). There is evidence that the data, unlike previous studies, were devoid of pre-analytical biases (120).

9. SERUM NEUROENDOCRINE MARKERS IN PROSTATE CANCER

The measurement of serum neuroendocrine markers constitutes a more representative indicator and more objective quantification of significant neuroendocrine differentiation of tumors, as it corresponds to the entire primary tumor cell population and its associated metastases (121). The majority of neuroendocrine products can be released into the blood stream and measured using immunoassay techniques. Out of the neuroendocrine markers, Chromogranin A (CGA) and Neuron Specific Enolase (NSE) are commonly expressed in neuroendocrine prostatic carcinoma. Recently, was reported that pre-treatment serum neuroendocrine markers such as CGA, NSE and Pro-gastrin releasing peptide (Pro-GRP) were (NED) in prostatic tumors. (128). Due to improved measuring techniques, it has become easier to measure serum or plasma CGA and the results have become more reliable. Wu *et al* reported that CGA should be used as a marker for NED and an early elevated serum level indicates resistance to hormone therapy (129).

Kadmon *et al.* reported that 48% of patients with stage D2 Prostate cancer had elevated serum CGA and the level in some closely paralleled the clinical course. (130). Wu *et al.* noted that serum CGA was elevated in patients who did not undergo hormone therapy and the serum level enabled the early detection of hormone therapy resistance in Prostate cancer, although the serum concentration in those with BPH overlapped considerably with that of men with Prostate cancer (129). Isshiki *et al.* have shown that mean serum CGA in Prostate cancer and benign prostatic hyperplasia cases was 59.4 and 59.3 ng/mL, respectively (no significant difference); however, poorly differentiated adenocarcinoma was associated with higher CGA than well

differentiated disease (P = 0.044) (122). More importantly in the same study, of the stage D cases with a median PSA of 172.1 ng/mL or less, those with higher CGA had a poorer prognosis than those with lower CGA. Therefore, CGA in combination with PSA may effectively predict a poor prognosis after hormone therapy in stage D2 Prostate cancer.

9.2. Neuron Specific Enolase (NSE)

Circulating NSE in caP was also studied as CGA. A few have failed to demonstrate any clinical significance of serum NSE level in the pathogenesis of prostate cancer. Tale *et al.* examined serum concentrations of NSE in Prostate cancer patients and suggested that NED, as reflected by an increase in serum concentrations of these NE secretory products, correlated with androgen independence and poor prognosis (131). In addition, another study reported that elevated NSE and CGA levels were predictors for poor prognosis in patients with hormone-refractory prostate cancer, whereas CGA appears to reflect the NE activity of Prostate cancer rather than NSE. Kamiya *et al.* demonstrated that poor survival in metastatic patients with higher NSE level and no significant correlation among serum levels of PSA, NSE and CGA (132). Multivariate analysis of cause-specific survival in patients with metastatic Prostate cancer, serum NSE showed the highest risk (18.3, P = 0.0064) for poor survival after hormone therapy among other variables including extent of disease of bone metastasis (relative risk 10.3, P = 0.029), serum CGA (8.72, P = 0.024), serum PSA, histological grade, response to hormone therapy (latter three were not significant) (133). Therefore, serum NSE can be the strongest prognostic factor for metastatic prostate cancer hormonally treated. When both of the serum levels of NSE and CGA were combined, patients with elevated NSE as well as CGA had the worst survival as compared with the others. Their survival was less than 27 months.

9.3. Gastrin-releasing peptide (GRP)

Gastrin-releasing peptide (GRP), a mammalian homolog of the amphibian peptide bombesin, is composed of 27 amino acids and is widely distributed throughout the mammalian nervous system, gastrointestinal tract, pulmonary tract, and prostatic neuroendocrine cells (134). GRP is initially synthesized as amino acids 1–27 of a 125 residue precursor, ProGRP, and is subsequently cleaved and amidated to form GRP18-27. ProGRP has a longer half-life than GRP and is detectable in serum at levels similar to those of GRP itself. This enables detection by means of a clinically applicable ELISA kit (135). Serum levels of ProGRP are extensively measured in the studies of lung cancer patients (136,137). From the finding of recent reports, serum ProGRP is also thought to be a useful diagnostic and therapeutic marker for Prostate cancer.

Nagakawa *et al.* and Yashi *et al.* measured its level in patients with Prostate cancer (138, 123). The mean serum levels of ProGRP in patients with distant metastasis and hormone-resistant prostate cancer were significantly elevated compared with those in patients with organ-confined disease. Yashi *et al.* demonstrated that elevated serum ProGRP level is a predictor of short response duration after hormonal therapy in metastatic Prostate cancer (139). A weak positive correlation between serum PSA and ProGRP values was found (rs = 0.268, P = 0.0311) when patients with pure NE carcinoma were excluded.

10. FUTURE PERSPECTIVE

Many authors are still researching novel biomarkers to improve our ability to early detect prostate cancer and foretell the course of the disease. New treatment modality (chemoprevention, gene therapy, adjuvant therapy, will need more reliable markers. We discussed about molecular forms of PSA and other promising urinary and serum markers: industries and researchers have recognized the utility and importance of biomarkers and are making efforts in this way. There is a disjunction between the multiple novel biomarkers investigated and the clinical practice. For new biomarkers to be clinically useful, they must answer at clinically relevant questions and provide information that is not available in a simpler and more cost-effective way. Despite their scientific value or validity the novel biomarkers should provide additional information that is helpful to the clinician for the management of the disease. New biomarkers assays should also be able to be performed easily and promptly in clinical environment. To this end biomarkers should be performed on multiple platforms that can perform multiple assays; obviously, the assay should also be valid, reproducible and should give the information requested in an efficient and timely manner: even if the new markers have been proven to offer valuable information regarding the disease, an unreasonable period of time for its delivery would considerably decrease its interest. It is essential for a biomarker to be also cost effective. With health care expenditures reaching record levels, medical decision-making is increasingly affected by economic concerns. Many parameters must be considered when assessing the economic impact of a biomarker: cost of the assay, potential benefits of the assay (avoidance of ineffective therapy, benefit from targeted therapy), and positive/negative predictive values of the assay.

Potential savings of a new biomarker can be tremendous, particularly when dealing with newly released drugs that are very costly and might take several cycles of administration before any objective response is seen. However, it is most unlikely that a single biomarker will have the single decision as to a diagnosis or a prognosis of prostate cancer. It may rather be that a constellation of markers will have more predictive power.

The decrease in death from prostate cancer serves as indirect evidence that early detection saves lives: nowadays the only effective diagnostic tool to detect prostate cancer is biopsy: the preciousness of developing additional serum tumour markers which improve early detection and reduce the number of unnecessary biopsies would be invaluable. Several new markers have shown promise in early-phase biomarker studies: probably the way is an accurate molecular staging that could indicate the

likelihood of cancer presence, disease stage, metastasis and the need for targeted systemic therapy.

The development of simple diagnostic kits that will accurately and reliably predict cancer presence and biological behavior remains a crucial goal for the future of urological oncology.

11. AKNOWLEDGMENTS

The authors thank Dr. Vincenzo Favilla that contributed to the acquisition of data in this review

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Abbreviations: : AMACR: Alpha-methylacyl CoA racemase; caP: prostate cancer; PSA: prostatic specific antigen; PSMA: prostatic surface membrane antigen; GSTP-1glutathione S-transferase.

Key Words: Prostate cancer, markers, PSA, PCA3, EPCA, neuroendocrine markers, DNA biomarkers, prostate antigens, PSMA, Review

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