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Molecular Alterations in Prostate Cancer as Diagnostic, Prognostic and Therapeutic Targets

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Abstract

Prostatic adenocarcinoma is extremely common in Western nations, representing the second leading cause of cancer death in American men. The recent application of increasingly sophisticated molecular approaches to the study of prostate cancer in this “post-genomic” era has resulted in a rapid increase in the identification of somatic genome alterations as well as germline heritable risk factors in this disease. These findings are leading to a new understanding of the pathogenesis of prostate cancer and to the generation of new targets for diagnosis, prognosis, and prediction of therapeutic response. Although we are still in the very early phase of clinical development, some of the molecular alterations identified in prostate cancer are being translated into clinical practice.

The purpose of this review is to update the practicing surgical pathologist, and residents-in-training in pathology, regarding recent findings in the molecular pathobiology of prostate cancer. We will highlight some of the somatic molecular alterations associated with prostate cancer development and progression, with a focus on newer discoveries. In addition, recent studies in which new molecular diagnostic approaches have been applied in the clinic will be discussed.

Keywords

Prostatic adenocarcinoma; molecular pathology; hypermethylation; GSTP1; PCA3; urine

Epidemiology of Prostate Cancer

The major risk factors for the development of prostate cancer are advanced age, race (African Americans have the world’s highest rates), inherited susceptibility and environmental factors such as diet. In terms of diet, vitamin E, lycopene (or other carotenoids found in tomato based products) and selenium may exert a protective effect, while diets rich in fat and red meat, especially well-done meats, may exert a promotional effect[1-4]. Since each of the dietary factors that appear to protect against prostate cancer are potent antioxidants, it is widely held that oxidative stress (which can directly damage DNA)

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may contribute to prostate carcinogenesis. Potential sources of oxidant stress are endogenous metabolism, inflammation and dietary factors. Circulating levels of insulin like growth factor 1 (IGF-1), which can be influenced by diet or genetics, have been implicated in the development of aggressive prostate cancer[5].

Many, albeit not all, prostate adenocarcinomas are believed to be derived from high grade prostatic intraepithelial neoplasia (PIN)[6]. Over the last several years, our group has been working on a model whereby focal atrophy lesions, which are extremely common in the prostate and occur as a number of morphological variants, result from cellular injury imparted by dietary and inflammatory insults[7]. These atrophy lesions show morphological transitions to high grade PIN lesions, and at times directly to "microcarcinoma"[8] lesions. They also show clear evidence of a stress response, and some also contain molecular alterations that are generally found at much higher frequencies in high grade PIN and carcinoma lesions. Thus, certain atrophy lesions, which are often found in association with chronic inflammation, may be "risk factor lesions" for the development of PIN and adenocarcinoma of the prostate. One of the potentially important aspects of this research is that since inflammation and diet are known to play a prominent role in the development of cancer in many other organ systems[9, 10], they may become targets for the deployment of novel prevention strategies for prostate cancer.

Somatic Molecular Alterations in Prostate Cancer

Prostate cancer cells, like other cancer cells, usually contain a large number of somatic genome alterations[11-15] that contribute to the cancer phenotype. Some of the somatic alterations are genetic (changes in DNA sequence), such as point mutations, deletions, amplifications, and translocations. Other changes are epigenetic[1], including modifications in deoxycytidine methylation patterns and chromatin structure. A major challenge for researchers has been to decipher which changes are causal in the disease process and which occur as bystanders unrelated to disease pathogenesis.

Except for telomere shortening[16, 17] (a genetic change), somatic hypermethylation of deoxycytidine residues within CpG dinucleotides in the upstream regulatory regions of a number of genes occurs earlier and more consistently in prostate cancer than recurrent genetic changes do. Several of the genes silenced by epigenetic alterations have been identified, providing new potentially useful molecular biomarkers of prostate cancer and insights into prostate cancer etiology, and some of these will be discussed briefly below.

Genes Silenced by CpG Island Hypermethylation in Prostate Cancer

GSTP1—*GSTP1* encodes the π-class Glutathione S-transferase (GST-π). GSTs are an enzyme family that can detoxify reactive chemical species by catalyzing their conjugation to reduced glutathione. Thus, *GSTP1* likely serves as a "caretaker" gene, defending prostate cells against genomic damage mediated by carcinogens or various oxidants. Loss of *GSTP1* function may render prostatic cells sensitive to carcinogenesis driven by inflammation and dietary factors.

Since the first study of CpG island hypermethylation within the *GSTP1* promoter region[18], a large number of studies have verified this finding[19] which occurs in over 90% of prostate cancers[19-21].

[1]The term epigenetic refers to changes in a cell's phenotype that are stably inherited through cell division but have not resulted from a change in DNA sequence. Epigenetic mechanisms include methylation of deoxycytidine residues within CpG dinucleotides, histone modifications such as methylation and acetylation, RNA interference and others.

Other Genes Methylated in Prostate Cancer—A number of other genes have also been found to be hypermethylated in prostate cancer[21-25]. Using quantitative real-time methylation specific PCR (Real Time-MSP), Yegnasubramanian et al. assessed the extent of hypermethylation in 16 different genes in prostate cancer and found strikingly high frequencies of hypermethylation in the CpG islands associated with *GSTP1*, *APC*, *RASSF1a*, *PTGS2* and *MDR1*, but virtually no methylation in normal prostate tissues[22].

While hypermethylation of specific genes is likely to be useful diagnostically and perhaps prognostically (see below), how these methylated genes may be etiologically involved in prostatic carcinogenesis remains unclear.

Somatic Genetic Alterations and Prostate Cancer

Like other cancer types, prostate cancers often contain genetic changes at the chromosomal or sub-chromosomal level[11-15, 26-29]. The most common chromosomal abnormalities are losses at 8p, 10q, 13q, 16q, and the recently described recurrent losses and rearrangements on chromosome 22q between the TMPRSS2 and ERG gene loci. Recurrent gains include those at 7p, 7q, 8q, and Xq.

Telomere Shortening—Telomeres are composed of repeat DNA sequences bound to specific binding proteins at the termini of chromosomes. Telomeres serve to protect against loss of chromosome sequences and illegitimate recombination between chromosome arms or at DNA double strand breaks. Telomerase is a multi-component enzyme that acts to extend telomere sequences in order to maintain chromosomal length despite loss of telomeric sequences due to the “end replication problem”. Telomeres become markedly shortened during the development of most cancers, most likely to the point where chromosomal instability ensues[30]. Mice carrying disrupted genes encoding telomerase subunits show increased numbers of cancers, especially when crossed to mice with deleted *p53* genes[31]. In the human prostate, somatic telomere shortening occurs in the luminal cells of the vast majority of cases of high grade PIN and carcinomas[16, 17]. At the same time, prostate cancers[32] and some PIN lesions[33] paradoxically show telomerase activity, whereas normal prostate tissue and BPH do not. Thus, telomere shortening may be a nearly universal feature of early prostate cancer and may promote chromosomal instability leading to disease progression. While the telomere FISH assay is generally used to demonstrate that telomeres are short in tissue sections, recently Meeker et al. have developed a chromogenic in situ hybridization approach that may prove useful in applications of prostate biopsies or other specimens in the clinic[34].

Selected Tumor Suppressor Genes and Loss of Heterozygosity (LOH)—Deletions of genomic sequences from sites on chromosome 8p occur frequently in prostate cancer[35]. Loss of 8p appears to be an early event since high grade PIN may show LOH at this location[35], albeit the fraction of high grade PIN lesions with this change may be less than previously thought[36]. Several genes located on chromosome 8p have been examined as candidate tumor suppressors, with one of the most promising being *NKX3.1*.

The product of the *NKX3.1* gene, which is a prostate restricted homeobox protein that is involved in the regulation of prostate development, is expressed in normal prostate epithelium and is often decreased in PIN lesions and in prostate tumor cells[37, 38]. Further, mice lacking either one or both *Nkx3.1* alleles develop abnormal prostate ductal branching, prostatic hyperplasia and lesions similar to human PIN[38-40]. NKX3.1 protein has also been implicated in helping to decrease oxidant genome damage by virtue of its ability to activate expression of genes involved in scavenging oxidant radicals[41], and we have found a profound decrease in prostate atrophy lesions and PIN lesions, as compared to normal

prostatic epithelium[36]. *NKX3.1*, however, may not be the only target for deletion in this region, since chromosome 8p is also deleted frequently in other cancer types, such as those of the colon/rectum, and these other tissues do not express *NKX3.1*. In addition, most prostatic adenocarcinomas, even those that are very high grade[42] or metastatic, still express significant levels of NKX3.1 protein indicating that *NKX3.1* is clearly not a classical tumor suppressor gene. The fact that NKX3.1 is expressed in most prostate cancers, and not in most other tumor types, suggests that NKX3.1 may be an excellent immunohistochemical marker of prostate cancer[43].

In a recent study, Chang et al[28]., used high-resolution Affymetrix SNP arrays to define detailed deletion patterns at chromosome 8p and reported two commonly deleted small regions at 8p21.3 and 8p23.1 and that these same regions showed evidence for linkage to hereditary prostate cancer patients. These relatively small “consensus” regions will likely facilitate more effective searches for prostate cancer genes that may be located on chromosome 8p, perhaps by large scale DNA sequencing analyses in this region.

The *PTEN* gene on 10q23 is mutated in up to 1/3 of hormone refractory prostate cancers[35] and homozygous deletions and mutations have been identified in a subset of primary prostate cancers[35, 44]. Loss of PTEN protein in primary prostate cancer, as determined by immunohistochemistry, correlates with high Gleason score and advanced stage[45]. PTEN is a dual protein and lipid phosphatase that is responsible for dephosphorylation and inactivation of phosphatidylinositol 3,4,5-trisphosphate (PIP3), a second messenger that is produced after activation of PIP3 kinase in response to ligation of several growth factor receptors, including IGF-1. PIP3 activates the protein kinase AKT. AKT signalling results in inhibition of apoptosis in response to a variety of signals and to increased cell proliferation[46].

Another potential role for AKT related to prostate cancer is the finding that AKT can phosphorylate p27 ^{Kip1} protein, resulting in cytoplasmic retention of p27^{Kip1} and lack of p27 ^{Kip1} mediated cell cycle arrest [47]. Levels of p27^{Kip1}, encoded by the *CDKN1b* gene, are often downregulated within the nucleus of prostate cancer and high grade PIN cells. Inactivation of p27 ^{Kip1} cannot, however, be the only function of the PTEN pathway during prostate carcinogenesis; in the mouse, *PTEN* can cooperate with either *NKX3.1* or *CDKN1b* (encoding p27^{Kip1}) in increasing the frequency and extent of high grade PIN lesions and perhaps early cancers[48, 49]. Since this pathway is commonly altered in prostate cancer, inhibition of signalling through PI3K and AKT is a promising therapeutic strategy in this disease[46, 50].

Other sites of loss/deletion in prostate cancer mainly occur in the late stages of cancer progression. Genetic inactivation of the classic tumor suppressor genes *p53*, *RB1*, *p16*, are seen rarely in primary cancers, but occur at higher frequencies in metastatic and/or hormone refractory lesions[35], suggesting that these genes may be involved in prostate cancer progression.

Selected Gene Targets in Regions of Chromosomal Gain—High-level amplification of the *ERBB2* gene (often referred to as HER2 or HER2/NEU) does not occur in prostate cancer to any great extent[51]. However, amplification of certain regions on chromosome 8q correlates with aggressiveness of tumors[52-54]. One candidate for amplification on 8q is the *C-MYC* oncogene (see more on *C-MYC* below). Another gene on chromosome 8q that is often amplified in prostate cancer is *PSCA*, encoding prostate stem cell antigen, which is also accompanied by demonstrable corresponding protein overexpression[55-57]. *PSCA* is a cell surface marker, and humanized antibodies or fragments thereof are currently being investigated in clinical trials in patients with metastatic prostate cancer[58]. Other genes on chromosome 8q have also been implicated recently as potential

targets of amplification, including the *Elongin C* gene[59] and the *EIF3S3* [60] gene. Other regions of gain include the *AR* gene itself (located on Xq12), where amplification occurs almost exclusively in the hormone refractory state[61].

Selected Oncogenes/Growth Promoting Genes in Prostate Cancer

Androgen receptor: The prostate requires androgenic hormones and an intact androgen receptor (AR) for normal growth and development. In the normal prostate, AR is expressed highly in the luminal epithelial cells where it is present largely within nuclei. Much lower levels of AR are expressed in prostatic basal epithelial cells, and many prostatic stromal cells also contain nuclear AR. Luminal cells in high grade PIN and the vast majority of prostatic adenocarcinoma cells express AR at relatively high levels. Metastatic prostate cancer is almost always treated with androgen deprivation, anti-androgens, or a combination of the two. However, despite such treatment, "androgen-independent" prostate cancer cells eventually emerge. Despite their apparent androgen independence, however, in most hormone refractory prostate cancers, androgen-receptor (AR) expression and AR signaling remain intact[62] and AR is critical for androgen-refractory prostate tumor cell proliferation[63]. In fact, AR expression itself is often increased in hormone refractory prostate cancer[64].

Somatic alterations of AR have been reported for many prostate cancers, especially for androgen-independent prostate cancers, and these mutations are often "activating" mutations[65]. AR mutations can also result in altered ligand specificity in which even anti-androgens can act as agonists[65]. In addition, AR gene amplification, accompanied by high-level expression of AR mRNA and protein, may promote the growth of androgen-independent prostate cancer cells by increasing the sensitivity of the cells to low androgen levels.

In addition to somatic AR gene changes, androgen-independent prostate cancer cells with wild-type AR may activate AR signaling even in the absence of androgens, through post-translational modifications of the AR and/or AR coactivators in response to other growth factor-signaling pathways[65].

While there is abundant data indicating AR can adapt to function in the setting of very low androgen levels, recent studies have suggested that prostate cancer cells may manufacture androgens themselves[64, 66]. Thus, AR signaling may be intact as a result of relatively high local androgen levels in the tumor microenvironment, despite castrate levels of androgens in circulation.

Oncogene Addiction and Lineage Survival in Prostate Cancer

Another emerging concept that may be related to AR in prostate cancer is the notion of oncogene addiction--the dependence of a cancer cell on one overactive gene or pathway for the cell's survival and growth[67, 68]. Evidence for this concept stems from a number of mouse models, and, in human cancers such as: (i) chronic myeloid leukemia or gastrointestinal stromal tumors treated with imatinib (Gleevec®); (ii) lung cancers containing epidermal growth factor receptor mutations treated with gefitinib (Iressa®) and erlotinib (Tarceva®); and (iii) *ERBB2* amplified breast cancers treated with Herceptin (Trastuzumab).

While AR is not a classical oncogene, evidence certainly indicates that prostate cancer cells that express AR are indeed "addicted" to AR signaling. Along these lines is the concept of "lineage dependency"[69] which suggests that "master regulator" genes, such as AR in the prostate, when deregulated in certain contexts can become an oncogene. This implies that as a function of their prior lineage development in the prostate, prostate cancer cells may be "hardwired" to use AR signaling for growth and survival in a manner that generally cannot

be later bypassed. Another way to look at this is that during the process of transformation of a normal prostate epithelial cell into a tumor cell, all of the genomic and epigenomic changes that drive prostate cancer growth, prevent apoptosis, induce angiogenesis, etc, occur in cell that has been epigenetically "programmed" to use androgen receptor signaling. Therefore, without AR signaling, these oncogenic changes are not tolerated by the cells. If correct, this hypothesis implies that the cell of origin (tumor stem cell or tumor progenitor cells) in prostate cancer is an AR positive cell and not likely an AR negative "stem cell". Clearly, the androgen receptor is still a major therapeutic target in prostate cancer and new ways to inhibit its function are continually under development[70].

C-MYC—The C-MYC protein is a nuclear transcription factor that regulates a number of cellular processes including cell cycle progression, metabolism, ribosome biogenesis, protein synthesis and mitochondrial function[71]. C-MYC is over-expressed in a large variety of tumor types, often associated with somatic genetic alterations such as translocations and gene amplification[72]. In prostate cancer, there is evidence that C-MYC is involved in disease progression since a region encompassing the *MYC* locus (8q24) is somatically amplified at low levels in a subset of patients[52, 72-74], and the presence of amplification in this region correlates with both high histological grade and worse prognosis[52-54, 73]. Whether there is amplification of *MYC* in high grade PIN is controversial since *MYC* amplification has been reported in up to 50% of HGPIN lesions[74], but more recent experiments revealed a lack of *MYC* amplification in such lesions[36].

It has been long known that a subset of prostate cancer lesions express elevated levels of *MYC* mRNA, often in parallel with increased expression of *PIM-1*, a gene known to cooperate with *MYC* in other malignancies[15], and that is often overexpressed in prostate cancer[75, 76]. Further, targeted overexpression of the human *MYC* gene in the mouse prostate results in PIN[77, 78], early invasive prostate adenocarcinoma[78] and rare metastatic adenocarcinoma[78]. These findings provide definitive evidence that C-MYC overexpression can drive neoplastic transformation in the mouse prostate, and support a model whereby C-MYC may play a role in initiation of human prostate cancer.

Nevertheless, due to a lack of suitable antibodies that can be readily applied for cellular and sub-cellular localization in archival tissues, the phase of prostate cancer development in which C-MYC protein is expressed in humans has been unclear.

In very recent work from our laboratory[79], using genetically-defined control experiments, we found strong nuclear staining for C-MYC in the majority of human clinical prostate cancer and high grade PIN samples, and much less staining in benign tissue. Although the levels were somewhat lower than Gleason score 6 tumors, high grade lesions (Gleason score 7-9) and hormone naïve metastatic lesions also showed marked overexpression in most cases for C-MYC protein. These new results suggest that the view of this key oncogenic transcription factor in prostate carcinogenesis should be revised to include activation in the majority of cases at a very early time point in the neoplastic process.

Gene Fusions in Prostate Cancer—Using transcriptome profiling of laser capture microdissected prostate cancer cells in a paper published in May of 2005, Petrovics et al., found that the most commonly overexpressed gene was the product of the v-ets erythroblastosis virus E26 oncogene like (*ERG*) gene[80]. *ERG* and other members of the *ETS* family had already been established as proto-oncogenes in other tumor types. For example, chromosomal translocations involving ERG are linked to Ewing sarcoma, myeloid leukemia and cervical carcinoma, and overexpression occurs in acute myeloid leukemia[81]. The absolute levels of *ERG* transcripts were found to be higher in moderate grade prostate cancer (Gleason scores 6-7) yet somewhat lower in higher grade cancer (Gleason scores 8-9).

Also in 2005, Arul Chinnaiyan and colleagues reported on a series of related discoveries that have begun to transform prostate cancer research. Tomlins et al.[82] used a novel analysis method, cancer outlier profile analysis (COPA), to discover genes with marked overexpression in a subset of prostate cancer cases. Starting with clinical prostate cancer specimens, COPA identified outlier profiles for *ERG* and ets variant gene 1 (*ETV1*), two ETS family transcription factors. Strikingly, the group showed the presence of aberrant mRNA transcripts containing sequences from the 5 prime region of the androgen regulated gene, *TMPRSS2*, that were fused to 3 prime exons from the *ERG* or *ETV1* genes. By using multiple approaches, including FISH to detect rearrangements of *TMPRSS2:ERG* loci, the Chinnaiyan group and others have concluded that rearrangements in ERG or other ETS family members occur in the majority of all PCA cases[15, 83](current estimates vary between ~40-70%). The same group has since shown that other ETS family members and other androgen regulated genes, or even housekeeping genes[84-86], may also be involved in gene fusions in prostate cancer. A number of other groups have verified these overall findings[29, 87-91], and some studies now indicate that *TMPRSS2:ERG* gene fusions can be identified in a subset of high grade PIN lesions[91, 92]. These gene fusions, therefore, have become a prime target for the development of novel diagnostic, prognostic, predictive and therapeutic approaches in prostate cancer. Of special interest for surgical pathologists, Mark Rubin and colleagues have recently shown that some of the morphological features of prostate cancer correlate with the presence of the *TMPRSS2:ERG* rearrangement[93].

Currently the prognostic significance of these gene fusions is uncertain. Some studies suggest that the presence of fusion transcripts, or altered types or numbers of copies of the fusion[89, 94-96], portend a worse prognosis. Other data, however, do not support this hypothesis and in fact suggest higher levels of *ERG* mRNA are associated with an improved prognosis[80].

Given the excitement regarding these gene fusions, it is certain that a great deal of new information on this subject will be generated over the next few years, and the challenge will be to discover ways to implement these findings into clinical practice (see below).

Approaches to Using Molecular Alterations as Early Diagnostic Markers

There are several major challenges in prostate cancer care in which molecular markers are expected to become highly useful in the clinic. These include: (i) early detection, including the determination of who may or may not require an initial prostate biopsy, and, who may require rebiopsy after an initial negative biopsy; (ii) monitoring of low risk prostate cancer patients who do not elect immediate treatment and are undergoing “active surveillance”; (iii) prediction of recurrence after initial treatment to stratify patients into risk groups for emerging adjuvant therapies; (iv) detection of recurrence after treatment; and (v) development of surrogate markers for assessing the efficacy of treatments in advanced disease. Most efforts to apply non-prostate specific antigen (PSA) related molecular markers have so far have been in the area of early detection, so early detection will be the focus of the next part of this review.

Applications to Urine or Post-Prostate Massage Urine

Most prostate cancers are now discovered by transrectal prostate needle biopsy in men who are found to have elevated serum PSA levels (usually ≥4 ng/mL, but often as low as 2.5 ng/mL). Several groups have been attempting to examine urine specimens for molecular alterations associated with prostate cancer in order to improve upon the ability of serum PSA to predict the presence of prostate cancer. This is an important area of research since elevated serum PSA levels can be reasonably sensitive for prediction of prostate cancer in a needle biopsy (~80%), but at a cost of poor specificity (~25-40%). In addition, the negative

predictive value of a low serum PSA is also not very robust. As reported in the Prostate Cancer Prevention Trial (PCPT) when men without an elevated PSA or abnormal digital rectal exam underwent random prostate needle biopsy, the prevalence of prostate cancer was 6.6 percent among men with a PSA level of up to 0.5 ng/mL, 10.1 percent among those with values of 0.6 to 1.0 ng/mL, 17.0 percent among those with values of 1.1 to 2.0 ng/mL, 23.9 percent among those with values of 2.1 to 3.0 ng/mL, and 26.9 percent among those with values of 3.1 to 4.0 ng/mL[97].

It should be also be noted that a major limitation of all studies designed to improve upon PSA testing is that the gold standard (prostate biopsy) is still approximately only 80-85% sensitive. This reflects the reality that prostate needle biopsies are typically carried out in a blinded fashion and may miss cancer in up to 15-20% of patients. Therefore the test performance parameter measurements (sensitivity, specificity, and positive and negative predictive values) of tests designed to predict the presence of prostate cancer by testing for molecular markers in bodily fluids or serum are necessarily only relevant to the prediction of a positive prostate needle biopsy, and not specifically to the presence or absence of cancer.

Nevertheless, one major application has been in attempts to determine which patients with elevated serum PSA actually need a biopsy. Given the false negative rate of prostate needle biopsies, another way to state this is to ask: can the molecular marker help to determine which patients have a high risk for the finding of cancer on a prostate needle biopsy? The goal of such a test is to prevent “unnecessary biopsies”.

DNA Markers—As mentioned above, methylation of deoxycytidine residues within CpG islands in the upstream regulatory regions of a number of genes occurs in a very high percentage of prostate cancers and is not found to any significant extent in normal prostate tissues in most studies. Therefore a number of groups[98-103] have attempted to improve on the ability of serum PSA to predict a positive biopsy using methylation of *GSTP1* and other genes in the urine (as well as other bodily fluids), and a number of these studies have been reviewed.[19]

One of the first studies using DNA based tests was by Goessl et al.,[98] who used MSP to detect *GSTP1* hypermethylation in bodily fluids. Whereas *GSTP1* promoter hypermethylation was not detectable in prostate tissue and bodily fluids from patients with BPH, these authors reported that methylation was detected in 94% of tumors (16 of 17), 72% of plasma or serum samples (23 of 32), 50% of ejaculate (4 of 8) and 36% of urine (4 of 11) from patients with prostate cancer. Additionally, MSP identified circulating tumor cells in 30% (10 of 33) of prostate cancer patients.

Goessl et al[104]., also used MSP to detect *GSTP1* hypermethylation in urine sediments from patients after prostate massage and found an overall sensitivity of 73% and a specificity of 98%, although some of these patients had advanced prostate cancer.

Rouprêt et al., recently used a 10 gene MSP approach in which urine samples were obtained from 95 consecutive radical prostatectomy patients and from 38 age-matched males (controls) with no history of genitourinary malignancy, negative prostate biopsies, and with or without BPH[105]. Radical prostatectomy patients underwent prostate massage and the first urine stream was then collected. The authors reported a sensitivity of 86% and a specificity of 89% for the 10 gene panel[105].

Results of another very recent study have been reported by Woodson et al.[106], in which 100 men were referred for prostate needle biopsy due to increased PSA, abnormal DRE or related symptoms. In this study methylation of *GSTP1* in post-massage urine had a 75%

sensitivity and a 98% specificity for cancer. It is not clear why this latter study showed such high performance, but the results imply that perhaps the use of *GSTP1* alone will be valuable as a molecular marker in prostate cancer in urine specimens.

Until now all of the studies that have detected methylation of genomic DNA in bodily fluids (and serum–see below) for the detection of prostate cancer have relied on some form of methylation-specific PCR. One major limitation of this approach is that the DNA must be first treated with sodium bisulfite, which is a very harsh treatment and results in damage to what are often already low quantities of DNA. As a result, Yegnasubramanian et al., have developed a first generation assay, referred to as COMPARE-MS (combination methylated-DNA precipitation and methylation sensitive restriction enzymes) that does not rely on bisulfite treatment of DNA and this approach promises to increase the sensitivity of detecting CpG island hypermethylation[107]. The approach, which results in very high sensitivity and specificity, features fragmenting genomic DNA with restriction enzymes including restriction enzymes that only cut when the target sequence is unmethylated, capture of methylated DNA using a purified recombinant methyl-binding domain polypeptide fragment from the human MBD2 protein, followed by PCR for the gene of interest. The assay was found to be highly sensitive and specific[107]. It is anticipated that this type of approach may indeed improve upon existing approaches for both specific genes and for the ability to multiplex a number of genes.

RNA Markers—Another series of studies that have been employing molecular tests using urine to help predict prostate cancer has used RNA-based approaches. Most studies have employed the RNA product of a gene originally named *DD3*[108] and now commonly referred to as *PCA3*[109]. *PCA3* is expressed nearly exclusively in the prostate, with much higher levels in prostate cancer, and it encodes an RNA product of unknown function that does not contain a protein coding open reading frame[108]. Hessels et al.,[110] studied post-prostate massage urine samples in men with elevated serum PSA (>3) and found, using a quantitative RT-PCR based approach to detect *PCA3*, that the sensitivity for prediction of a positive biopsy was 67%, with a negative predictive value of 90%. These results ultimately led to the development of a clinical test by Gen-Probe Inc. referred to as the APTIMA® PCA3 assay. This test uses whole urine specimens and includes target capture, transcription-mediated amplification, and a hybridization protection assay. In the initial study using this method, post prostate massage urines were obtained from 3 groups: men scheduled for prostate biopsy (n = 70), healthy men (<45 years of age with no known prostate cancer risk factors; n = 52), and men who had undergone radical prostatectomy (n = 21). ROC curve analysis showed an area under the curve of 0.746 and a sensitivity of 69% and specificity of 79%. In this study, the negative predictive value was 90%[111].

Fradet et al., used what was referred to as the uPM3 test in post-massage urines in a multicenter study in Canada enrolling 517 patients (of which 86% were informative) with elevated serum PSA and reported essentially similar findings and added the fact that the test added value at all serum PSA levels. The overall accuracy was 81% compared with 43% and 47% for total PSA at a cutoff of 2.5 and 4.0 ng/mL, respectively[112]. Schalken and colleagues used a second generation of this test based on RT-PCR, in a large multicenter Dutch trial consisting of 534 men, and, this study too showed very promising results[113]. A different commercial version of this urine test referred to as PCA3*Plus*®is offered currently by Bostwick Laboratories®. It should be noted that none of these urine based tests have been FDA approved for the diagnosis of prostate cancer, but that they are being offered so far as an aid to decision making regarding who should undergo a prostate needle biopsy or a repeat biopsy.

Laxman et al. showed the ability to detect *TMPRSS2*:*ERG* gene fusion transcripts in urine from prostate cancer patients[114], and, more recently added the detection of such transcripts to a multiplex RNA based assay that included *PCA3*[115]. In this study, which consisted of patients with known prostate cancer (n = 86 positive needle biopsies and 52 radical prostatectomy patients) and patients with negative needle biopsies (n = 96), the authors reported the area under the ROC curve was 0.758 for the multiplexed assay versus 0.66 for PCA3 alone. Another application of this type of test is to apply it to men that have already undergone a biopsy that was negative for cancer, but there is still clinical suspicion of prostate cancer. Marks et al., used the APTIMA®PCA3 test on men with a prior negative prostate biopsy but with a persistently elevated serum PSA of >2.5(ng/mL)[116]. Receiver operating characteristic curve analysis yielded an area under the curve of 0.68 for the PCA3 score and 0.52 for PSA. The assay sensitivity was 58% and specificity was 72%, with an odds ratio of 3.6.

An important potential limitation of most of the studies mentioned above is that the authors generally have not compared the predictive ability of their molecular markers to that of measurements of the relative levels of free to total serum PSA, which have been shown in a number of studies to improve the predictive ability of PSA in terms of identifying patients that will have a positive biopsy[117, 118].

Assessment of Molecular Markers in Tissues Remaining in Paraffin Blocks from Negative Prostate Biopsies—A number of groups have been attempting to determine whether assessment of methylation of *GSTP1* and/or other genes (e.g. APC) in DNA isolated from tissue remaining in paraffin blocks in samples, that were considered benign by pathologists, can aid in predicting a positive repeat biopsy[119]. In general, this approach appears to demonstrate a potential to avoid unnecessary repeat biopsies. Laboratory Corporation of America (Labcorp®) recently announced the availability of a commercial test using this approach. As with all of the other approaches that do not examine the cells directly under the microscope, it is not clear whether the assay is detecting cancer cells, high grade PIN cells, or rare methylated atrophic cells[120] that were not originally sampled by microtome sections, or, whether it is detecting a “field effect” whereby normal appearing prostate tissues harbor molecular alterations that are predictive of cancer on subsequent biopsies. In terms of *GSTP1*, a study using laser capture microdissection has shown that there is no methylation detected in normal appearing glands in any of the prostate zones[120]. Methylation was detected in a small subset of atrophy lesions (~6%), and in the majority of PIN (~70%) and adenocarcinomas (~90%)[120]. Thus for *GSTP1*, it would appear that what is being detected is likely to be unsampled carcinoma or PIN cells that remain in the paraffin block after standard histological sections have been obtained. In a preliminary study, *APC* methylation status appeared to perform better than *GSTP1* in predicting the biopsy results of a repeat biopsy in men with risk factors suggestive of cancer (e.g. high serum PSA, previous PIN or atypical glands on biopsy), suggesting that perhaps *APC* methylation does occur in non-neoplastic cells more commonly in the prostate than *GSTP1* methylation and that when present it is a useful predictor of cancer[121].

Other Bodily Fluids and Serum

A number of studies have been performed that have also attempted to examine methylated DNA isolated from ejaculate fluid or from serum. Most of these studies have been used for prediction of prognosis and will be described below.

How Will The Adoption of These Novel Molecular Tests Affect The Practice Of Surgical Pathology?

At present it is not clear how a future potential widespread deployment of these types of molecular assays will affect the number of men undergoing prostate needle biopsy. For example, what if these tests become the gold standard for screening populations, replacing serum PSA? Since these tests are more specific than PSA, the overall number of men that are biopsied may decrease. Yet, it is not clear just how sensitive these tests will be in the population at large. At present it would appear that these tests are not likely to identify more small "insignificant" cancers than currently employed screening approaches do based on serum PSA (which is estimated to be approximately 20% of all cases in the United States). If these tests improve further in sensitivity, however, it is possible that they may indeed begin to identify more cancers than presently used strategies since it is estimated that more than 50% of men who are 50-75 years of age who are autopsied have microscopic prostate cancer lesions[122]. In the short term since these molecular tests are specifically designed to predict positive biopsies it would appear that the use of these tests will increase the fraction of needle biopsy sets that contain cancer.

Molecular Alterations as Prognostic Markers

A number of biomarkers have been studied (both in needle biopsy specimens and in radical prostatectomy specimens) in order to enhance the prediction of outcome in prostate cancer patients. Older studies have shown that ploidy status, immunohistochemical staining for markers such as Ki67, bcl-2, p53, and $p27^{Kip1}$, FISH analysis for chromosome 8q24 amplification, and nuclear morphometry measurements can add value to the prediction of outcome in prostate cancer patients. However, none are currently employed routinely in clinical practice. There may be a number of reasons for this. First, there is often a lack of inter-study reproducibility. This can either result from lack of standardization of measurements, variable study designs often employing small numbers of select patients, or simply a lack of a profitable market (i.e., the lack of adjuvant therapy for high risk prostate cancer patients results in a lack of need to stratify patients beyond the available clinic-pathological parameters) for the development of such tests.

In order to address whether a number of different markers can add value to the prediction of biochemical recurrence in patients undergoing prostate needle biopsy, a group of investigators from 11 National Cancer Institute funded prostate SPORE (Specialized Projects of Research Excellence Awards) programs have begun to accrue moderate to high risk patients with prostate cancer to a prospective study (n = 700). This study will hopefully determine whether selected markers applied to prostate needle biopsies may be clinically useful to predict outcome beyond typical clinic-pathological measurements such as Gleason score, serum PSA, number of cores positive, etc.

In terms of current use, the most promising marker right now is actually based on serum PSA itself. That is, serum PSA velocity or the PSA doubling time appears to be a very powerful predictor of disease progression, both after biochemical recurrence following primary treatment, and even when measured within 18 months prior to the initial prostate cancer diagnosis[123, 124]. In fact, the latter may indeed become a useful biomarker to better stratify patients with positive biopsies into risk groups such that more men may safely elect active surveillance as opposed to immediate treatment. Certainly a number of other molecular markers are under development that may be applied to serum for similar uses.

Role of Methylation Markers in Predicting Prognosis

A number of studies have begun to examine the ability of quantitative changes in DNA methylation, as measured either in prostate cancer tissues or in serum, to augment prediction of outcome for prostate cancer patients[22, 125-128]. Although large trials are needed before clinical implementation, several of these studies suggest that methylation markers may add value to existing models in predicting outcome in prostate cancer.

Molecular Alterations as Predictive Markers

Molecular markers that are expected to be widely used in the future are the so called "predictive markers" that help to stratify patients into groups that will likely respond to specific targeted therapeutic interventions. While this type of approach has been commonly used in breast cancer and more recently in lung cancer, it is also expected to become widely employed in prostate cancer care as well. The most promising pathway in which this is likely to be employed in the near future is the PTEN/PI3K pathway as a number of clinical trials using inhibitors of this pathway are in development or underway in prostate cancer[129]. Thus, the measurement of PTEN protein levels and downstream targets of AKT in prostate needle biopsies may have value in the future if these trials show promise[129].

What is on the Horizon for Prostate Cancer?

One the most promising areas that has been accelerated greatly by the human genome project is the development of methods to perform "Genome Wide Association Studies" (GWAS) in which disease risk is related to germ line polymorphic variants. These studies, which currently employ up to ~1,000,000 genetic makers referred to as SNPs (single nucleotide polymorphisms), are beginning to revolutionize medicine. Within the last year alone, a number of different research groups have identified regions on chromosome 8q24 (not within the *C-MYC* gene), and other novel loci, that harbor SNP variants that are associated with increased risk of prostate cancer[130-139]. What is striking about many of these new studies is that the reproducibility across different patient populations appears to be remarkably high. Another feature that is emerging from such studies is that it may be the combination of individual risk alleles that confers the most significant risk such that having only one or two risk alleles only confers moderately elevated risk but having a larger number of risk variants in one's gremline DNA confers a much greater risk[136]. Interestingly, at least one of these recent studies has resulted in the formation of a company that is developing tests that will attempt to provide patients with information regarding their genetic risk of prostate cancer. While this may not be ready for "prime time", it does appear that these types of tests will become highly popular in the future. In the short term, these findings point to new areas of research to attempt to decipher how these variants, which are often in non-protein coding areas and even in areas devoid of any known genes, influence prostate cancer risk.

Another area of research that has exploded onto the scene of cancer research is the study of microRNAs[140-143]. These non-protein coding small RNAs that are encoded by specific genes, are double stranded and range in size from 20-25 nucleotides in length in their mature form, were discovered in worms in the 1990s but have already revolutionized basic science research and are poised to change medicine soon. In fact, altered expression patterns of microRNAs are found commonly in cancer[144, 145]. It is expected that these molecules will become useful as diagnostic targets, therapeutic targets, and as therapeutic agents themselves.

Concluding Remarks

Although we are still at the very beginning phase of understanding prostate cancer at the molecular level, there is great promise for employing recent findings to some of the ongoing vexing problems in clinical practice. Nevertheless, translation of this new knowledge into new clinical tests that can ultimately better serve patients, at all phases of the disease process, is a daunting task. To accomplish this, there is a great need to conduct appropriately designed and sufficiently powered studies. These studies also need to be performed in conjunction with acquisition of well annotated biospecimens and detailed clinical follow-up information. Such studies require an integrated approach that includes investigators from multiple disciplines such as bench scientists, epidemiologists, biostatisticians/bioinformatics specialists, pathologists, urologists, medical oncologists, radiation oncologists and radiologists. What would appear to be “good news” for pathologists is that the widespread implementation of such tests will require highly experienced diagnostic pathologists/ laboratory medicine experts for both proper selection and interpretation of such tests.

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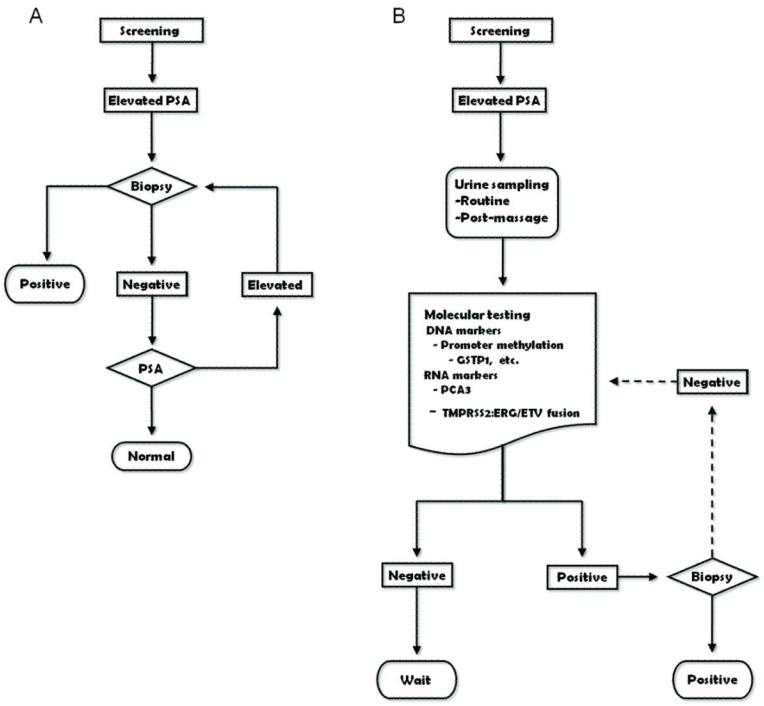

Figure 1.

(A) Current guidelines in the management of patients with elevated PSA at the time of screening. (B) The inclusion of molecular testing for prostate cancer markers may help in predicting a positive prostate biopsy, therefore reducing the number of patients that would otherwise enter an “elevated PSA, negative biopsy” loop (Dashed line).

Table 1

Urine-based studies testing for molecular alterations as early diagnostic markers.

	# Cases	Method	Sensitivity (%)	Specificity (%)	AUC	NPV
DNA-Based Tests						
Goessl et al.[105]	92	MSP for GSTP1 methylation	73	98		
Rouprêt et al.[102]	133	MSP for a 10-gene panel	86	89	0.74-0.86	
Woodson et al.[103]	100	MSP for GSTP1 methylation	75	98		
RNA-Based Tests						
Hessels et al.[107]	108	RT-PCR for PCA3	67	83	0.72	90
Groskopf et al.[108]	143	APTIMA for PCA3	69	79	0.74	90
Fradet et al.[109]	517	uPM3	66	89	0.86	84
van Gils et al.[110]	583	RT-PCR for PCA3	65	66	0.66	80
Marks et al.[115]	233	APTIMA for PCA3	58	72	0.67	

NPV: Negative predictive value. AUC: Area under curve for ROC analysis.