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The Epigenetics of pca Diagnosis and Prognosis: Update on Clinical Applications

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Abstract

Purpose of Review—There is a major deficit in our ability to detect and predict the clinical behavior of prostate cancer (PCa). Epigenetic changes are associated with PCa development and progression. This review will focus on recent results in the clinical application of diagnostic and prognostic epigenetic markers.

Recent Findings—The development of high throughput technology has seen an enormous increase in the discovery of new markers that encompass epigenetic changes including those in DNA methylation and histone modifications. Application of these findings to urine and other biofluids, but also cancer and non-cancerous prostate tissue has resulted in new biomarkers. There has been a recent commercial development of a DNA methylation-based assay for identifying PCa risk from normal biopsy tissue. Other biomarkers are currently in the validation phase and encompass combinations of multiple genes.

Summary—Epigenetic changes improve the specificity and sensitivity of PCa diagnosis and have the potential to help determine clinical prognosis. Additional studies will not only provide new and better biomarker candidates, but have the potential to inform new therapeutic strategies given the reversibility of these processes.

Introduction

Epigenetics refers to the stable transmission of cellular information due to a chemical modification of the DNA without disrupting the DNA sequence. Among these processes include DNA methylation, RNA interference, histone post-translational modifications (PTMs), and genomic imprinting. Changes in tissue epigenetics can alter gene expression generating malignant transformation by assisting in tumor invasion, angiogenesis, and cellular dedifferentiation and proliferation. The process of prostate carcinogenesis is not well understood and many of the molecular mechanisms contributing to its progression are poorly described. To date, in addition to genetic mutations acting as a foundation for cancer

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development, epigenetic changes are now included in the molecular pathogenesis of PCa. Among the most common and well-known epigenetic changes seen in PCa is DNA methylation, where an addition of a methyl group to cytosine adjacent to guanine within the DNA sequence occurs. DNA methylation can be either hypo- or hypermethylated leading to chromosomal instability, altered gene expression and subsequent malignant changes. Newer research developing histone modifications have been undertaken, but to date, the majority of epigenetic markers used in PCa are DNA methylation based.

Epigenetic alterations offer an explanation for prostate tumor biology. It is known that PCa develops throughout the prostate gland at multiple foci suggesting a 'field effect'. Alterations in DNA methylation and imprinting also occur with aging in the human prostate¹ and may underlie the susceptibility of the prostate gland for developing cancer.² Underlying factors that promote an epigenetic field effect are comprehensive and include inflammation, diet and other environmental insults.² Epigenetic as well as genetic changes clearly participate in the development and progression of PCa. In contrast to genetic changes, epigenetic alterations are reversible and are an area of increasing interest with regards to therapeutics. With the advent of the Confirm MDx test, DNA methylation markers are being commercially used for the diagnosis of PCa, and several have prognostic potential either alone or in combination with other types of molecular markers. We describe recent epigenetic findings in PCa and focus on how these molecular changes are being utilized diagnostically in PCa management.

DNA methylation markers for prognosis

DNA methylation is the covalent binding of a methyl group to the dinucleotide CG and is sometimes referred to as the 'fifth base'. Dense methylation at so called CpG islands leads to gene silencing by inhibiting access of transcriptional activators. This can induce chromatin condensation and an inactive state in conjunction with histone deacetylases (HDACs) and other chromatin remodelers. Conversely, decreased methylation throughout the genome (hypomethylation) is a hallmark of cancer and participates in genome instability and the activation of proto-oncogenes.

Correlating epigenetic changes to clinical data is essential to using these gene alterations as possible biomarkers for PCa diagnosis. An even more critical area is generating a molecular marker, from a biofluid (urine, serum) or biopsy that discriminates between indolent and clinically significant PCa prior to treatment. Roughly two decades ago, one of the first hypermethylated gene promoters to be discovered in PCa was Glutathione S-transferase PI (GSTPI) a gene involved in the detoxification of xenobiotics and oxygen radicals.³ This hypermethylation occurs in over 90% of primary PCa and 70% of prostatic intraepithelial neoplasia (PIN) lesions⁴. A recent review on the correlation of GSTPI methylation with cancer prognosis concluded there is no convincing evidence for the use of this gene in cancer tissues for predicting early disease outcomes.⁵

GSTPI assays have been developed for detecting PCa in serum and other fluid sources such as urine, ejaculate, and expressed prostatic fluid. As a prognostic approach, serum levels of GSTPI have been recently assessed as to whether they are associated with response and

survival in men with castrate resistant PCa (CRPC) treated with docetaxol, cabazitaxel, or mitoxantrone chemotherapy.⁶ Using PCR analysis, in a phase II cohort, increased methylated GSTP1 was a better predictor of overall survival than PSA changes after 3 months ($p=0.02$). This may reflect increased circulating tumor burden. Of note, the study validated their findings in a phase II cohort thereby increasing support for the use of GSTP1 methylation as a marker of chemotherapy response in CRPC. Its application to other types of CRPC systemic treatments has yet to be investigated.

A comprehensive assessment of emerging candidate markers for prognosis was recently published by Strand *et al.*⁵ These are beyond the scope of this discussion, however several loci warrant mentioning as being validated, widely studied and include needle biopsy tissue. Adenomatous polyposis coli (*APC*) a well-characterized tumor suppressor regulating *Wnt* signaling⁷, is frequently found to be methylated in PCa and other tumors.⁸ It has been extensively studied in PCa, but is one of the few loci investigated using diagnostic biopsy material and looking at death as an outcome. *APC* methylation was investigated using methylation sensitive PCR or MSP in PCa tissue samples from two large patient sets (total 462), and was a significant predictor of PCa specific death when both cohorts were combined (OR 1.49 95% CI 1.11–2.00).⁹ A smaller study also using biopsy specimens reported APC methylation predicted either BCR, metastasis and/or death in a multivariate analysis.¹⁰ In a recent meta-analysis of RASSF1A in 19 studies¹¹, hypermethylation was associated with Gleason score, PSA level, and tumor stage in tissue and presented evidence for its role in predicting BCR.¹² Other genes with promising earlier data include ABDHD9,¹³ PTGS2,¹⁴ EVX1¹⁵ and CDH13.¹⁶

A series of methylation loci, *PITX2*, *C1orf114*, and *GABRE-miR-452-miR-224*, as well as the three-gene marker panel *AOX1/C1orf114/HAPLN3* have recently been identified to hold independent prognostic value for biochemical recurrence in several large multicenter studies. *PITX2* encodes the paired-like homeodomain transcription factor 2, involved in the regulation of cell-type specific proliferation and is thought to be an upstream regulator of the insulin-like growth factor 1 receptor and the androgen receptor.¹⁷ *PITX2* generated a HR of 2.39 (95% CI 1.45–3.94) in a multivariate analysis validation study of 496 US and European patients.¹⁸ *C1orf114*, also known as *CCDC181*, has an unknown function and was identified initially on methylation arrays.¹⁹ A three gene methylation signature of *C1orf114*, *AOX1*, and *HAPLN3* performed better than individual genes on recent analysis.¹⁹ These validated genes hold promise, but involve retrospective radical prostatectomy specimens. Since predicting tumor outcomes before treatment is the ideal goal, generating this information from biopsy specimens should ideally be the tissue source of choice.

DNA methylation profiling and next-generation sequencing (NGS) have made it possible to evaluate methylation differences between PCa and benign prostate tissues at thousands of loci simultaneously. This has greatly accelerated the discovery of novel methylation markers. A recent analysis using methylation arrays applied these findings to clinical follow-up and found 69 methylation alterations that predicted biochemical tumor recurrence after radical prostatectomy²⁰. The DNA methyltransferases, DNMT3B1 and DNMT3B2, were overexpressed in these tumors and represent a potential underlying drug target. In another larger study involving 238 tissue samples,²¹ in a multivariate analysis adjusting for Gleason

score and tumor stage, 16 genes were found in multiple statistical analyses to separate recurrence vs. non-recurrence and several (*CRIP1*, *FLNC*, *RASGRF2*, *RUNX3*, and *HS3ST2*) were also among the candidates validated by pyrosequencing. In a unique analysis, Lin *et al* used enhanced reduced bisulfite sequencing to investigate methylation in primary and CRPC specimens with a neuroendocrine phenotype.²² This resulted in the identification of a panel of 13 gene-associated loci (*GSTP1*, *GRASP*, *TMP4*, *KCNC2*, *TBX1*, *ZDHHC1*, *CAPG*, *RARRES2*, *SAC3D1*, *NKX2-1*, *FAM107A*, *SLC13A3*, *FILIP1L*) confirmed in a small independent group that demonstrated increased DNA methylation with disease progression. Ultimately, combining genes will provide increased specificity and sensitivity over single gene approaches and it is likely gene panels will be the methodology of choice in the future. These newer approaches require further validation efforts, but highlight the promise of DNA methylation gene panels for predicting the prognosis of patients with PCa.

Histone alterations for prognosis

Epigenetic alteration of histones has received considerably less attention than DNA methylation in PCa. However, in recent years there has been increased interest in the association of histone post-translational modifications (PTMs) and PCa outcomes. Histone PTMs dictate chromatin structure that can promote activation or silencing of associated genes. Few studies have analyzed the association between PTM status and patient outcomes. One such study using IHC staining showed patients with increased H3K18Acetylation and H3K4diMethylation had a significantly higher risk of prostate tumor recurrence (Log-rank $P = 0.0076$)²³. Additionally, H4K12Acetylation and H4R3diMethylation were shown to correlate with tumor stage. A more recent study by the same group further showed that H3K9diMethylation significantly identified low-grade PCa patients at risk for tumor recurrence (Log-rank $P = 0.0043$)²⁴. These markers have experienced less clinical application due in part to the limitations in the technology available for analysis as immunohistochemistry is the only method used clinically at present, but provide a promising avenue for further development.

Underlying changes in PTMs are due to aberrant histone modifying enzyme activity. Due to the reversible nature of PTMs, histone modifying enzymes present an attractive therapeutic target. Several of these enzymes have been found to be altered in PCa including *HDAC1*²⁵, *SIRT1*²⁶, and *EZH2*²⁷. Synthetic and natural inhibitory drugs of these enzymes have already been identified²⁸. The identification of patients with altered PTMs may serve as a guide to target specific PTM enzymes in a personalized medicine approach in the future.

Urine based epigenetic markers for detection

Anatomically, urine comes within close proximity to the prostate gland as it passes through the prostatic urethra during micturition. Prostate cells are shed into urine and are made available for clinical analysis. When examined cytologically in urine, PCa cells are typically found only in patients that have clinically advanced disease.²⁹ Similarly important is that free DNA may pass through urine, in the absence of PCa cells, and also be detected in macrophages that phagocytize tumor cells.³⁰ Thus, it would seem intuitive to employ strategies using urinalysis to evaluate epigenetic changes that may suggest a diagnosis of

PCa. As in prostate tissue, DNA methylation can be identified in urine by a number of modalities such as methylation-specific polymerase chain reaction (MSPCR), bisulfite sequencing, methylation-sensitive single nucleotide primer extension (MS-SNuPE), and combined bisulfite restriction analysis (COBRA).³¹ Urine based methods hold promise for real-time, rapid detection and high-throughput analysis of clinical samples as well as offering a truly non-invasive way of detecting PCa.³²

The most widely studied epigenetic alteration in the urine to date is methylation of the GSTP1 gene. In an early study, Gonzalgo and colleagues assessed 45 post-biopsy urine samples in men suspected of having PCa and used PCR analysis to detect methylation at the gene promoter.³³ GSTP1 methylation was present in 39% of patients with PCa on biopsy demonstrating a sensitivity of 58%. In a recent meta-analysis of 22 published papers on GSTP1 hypermethylation in biofluids, the gene appears to have a high specificity (88–100%), but a low sensitivity for PCa detection in urine (19–84%) and serum or plasma (13–70%).³⁴ This lower sensitivity is likely due to the infrequent free cancer DNA or cells in these fluids.

Methylation of GSTP1 appears to perform better in combination with PSA or other methylation biomarkers. A larger analysis of hypermethylation in urine after prostatic massage, using PCR, was performed in 10 separate genes (*GSTP1*, *RASSF1a*, *ECDH1*, *APC*, *DAPK*, *MGMT*, *p14*, *p16*, *RARβ2*, and *TIMP3*).³⁵ The authors found that in 93% of patients with PCa, at least 1 gene was hypermethylated generating a specificity of 0.74. When distinguishing malignant from nonmalignant specimens, ROC analysis (0.74–0.86) demonstrated a combination of *GSTP1*, *RARβ2*, *RASSF1a*, and *APC* to be the best at discriminating cancer from noncancer. Finally, in a prospective study screening 337 urine samples, an investigational prostate cancer methylation assay (ProCaM™) analyzing *GSTP1*, *APC*, and *RARβ2*, had better predictive accuracy (AUC 0.73) than age, DRE, family history, serum PSA, and prior negative biopsy and improved the decision making process.³⁶ This assay has not been commercially developed as of yet.

There are a number of limitations and challenges when utilizing urine as a biofluid for epigenetic markers. The stability of biological markers can vary with temperature, pH and processing time that pose a challenge to urine analysis. Urine sediments including proteins, precipitates and crystals may entrap biomarkers altering their quantification. A major confounder is the variable presence of other cell types including inflammatory and bladder cells. We and other centers have found that epithelial cells of prostate origin make up only 10–20% of the sample and that PCa cells are exceedingly rare leading to the low sensitivity of many cancer markers³². Prostatic adenocarcinomas do not form functional ducts that connect with the urethra. A potential avenue for circumventing this reliance on the presence of cancer cells is by utilizing epigenetic changes found in normal epithelial cells associated with a cancer field effect. Urine testing for epigenetic changes has the potential to provide a biomarker that is cost effective and noninvasive. However, clinical results to date have not lead to the widespread adoption of these approaches for PCa detection in part due to the limitations mentioned.

Cancer detection using non-tumor prostate tissue

PCa development is associated with both a peritumor response (e.g. field defect), as well as an apparent change in the histologically normal appearing tissue within the peripheral zone at distance from tumor foci. These findings have triggered the development of several distinct new tests based on the analysis of existing histologically negative biopsy tissue. Although these new tests are invasive, they do utilize an existing resource (i.e. biopsy tissue).

Epigenetic changes within a field of susceptibility associated with PCa development was first recognized by Jarrard and colleagues in 1995.³⁷ This study found alterations in genomic imprinting existed not only in the tumor, but were widespread in the peripheral prostate tissues.³⁸ A recent extension of these studies compared DNA methylation alterations utilizing methylation arrays in the peripheral prostate from men with cancer to those without cancer.³⁹ Hypomethylation occurred more frequently than hypermethylation and changes were found at only 0.2% of sites analyzed suggesting they occur less frequently. Several of these probes were used to map the extent of these DNA methylation changes and it was similar both adjacent and distant (>1cm) to tumor foci suggesting a more widespread field defect. Another interesting aspect was the finding that several markers associated with higher grade tumors opening the possibility to detect lethal PCas from normal appearing biopsy tissue.

A subsequent study used the most promising markers in a training set of 65 tissues and a multiplex model was created using multivariate logistic regression analysis.⁴⁰ This model was externally validated in a blinded fashion using a set of 50 histologically negative biopsy specimens. Individual genes were all highly significant in differentiating men with cancer. Regression models incorporated individual genes (*EVX1*, *CAVI*, and *FGFI*) as well as gene combinations (*EVX1* and *FGFI*) to differentiate between non-tumor associated and tumor associated tissues from the original training set. In external validation, uniplex models incorporating *EVX1*, *CAVI*, or *FGFI* distinguished between TA and NTA biopsy-negative specimens (AUC of 0.702, 0.696, and 0.658, respectively; $p < 0.05$). A multiplex model (*EVX1* and *FGFI*) identified PCa patients with an AUC of 0.774 ($p = 0.001$) and a negative predictive value of 0.909. Thus, a widespread epigenetic field defect can be utilized to detect the existence of PCa in patients with histologically negative biopsies. With further validation, this marker combination (*EVX1* and *FGFI*) has the potential to decrease the need for repeated prostate biopsies, a procedure associated with cost and complications.

The ConfirmMDx (quantitative methylation assay) is a currently marketed test that is CLIA approved and marketed, but has yet to be submitted for FDA authorization. This tissue based assay relies on epigenetic alterations surrounding tumor foci (i.e. the 'halo' effect). The test evaluates methylation in 3 separate genes *GSTPI*, *APC*, and *RASSF1* and any abnormality in any of these genes prompts a positive test. An initial European study (MATLOC) study using negative core biopsies revealed on multivariate analysis the assay to be a significant predictor of repeat biopsy outcome (OR 3.17, 95% CI 1.81–5.53) with a negative predictive value of 90% (95% CI 87–93).⁴¹ A follow-up study in a US population validated these results and demonstrated a NPV of 88% and functioned as a significant multivariate

predictor (OR 2.69, CI 95% 1.60–4.51).⁴² The presence of atypia on pathology as a multivariate predictor was found also twice as frequently in cancer cases than in controls (OR 2.11).

These tissue assays help to separate men who are free of PCa from those who are more likely to harbor the disease and reduce the number of unnecessary repeat biopsies. However, several limitations arise when using tissue based assays including the impact of infection/inflammation, available tissue retrieved for assessment, and ability to distinguish high grade from low grade (clinically insignificant) lesions. *APC* methylation is associated with inflammation.⁴³ In addition, the performance of the ConfirmMDx assay over a specialized pathologists reading and finding of atypia in the specimen (which frequently prompts a recommendation of rebiopsy) does not appear to be significantly different from the ConfirmMDx assay in the recent DOCUMENT study.⁴² Finally, as noted above DNA methylation of genes changes with aging and this may have an impact on the signature being evaluated. Regardless, this application of epigenetics to diagnostics is one of the most developed and appears to be a promising area clinically.

Conclusions

Currently, many of the key components incorporated in the diagnostic algorithm for PCa are inaccurate and may lead to unnecessary prostate biopsies, overtreatment, and patient anxiety. In the ‘post-PSA’ era new biomarkers are urgently needed that inform the physician and patient about not only the presence of cancer, but the biologic potential. Epigenetics has the potential to answer both of these questions. To date, only one test is commercially widespread (ConfirmMDx) but a number of other promising approaches are in validation studies. Ultimately, the important question for any test being adopted is whether clinical decision making is altered through use of the test. A corollary is whether the test saves healthcare dollars. In a recent model looking at the impact of a tissue-based assay on healthcare costs for a commercial health plan covering 1 million individuals, a budget model projected a reduction in 1106 biopsies and savings of over \$500k dollars annually.⁴⁴ Thus, there is great economic potential for the use of molecular assays in modern medicine.

Several unique challenges should be considered when analyzing epigenetic biomarkers. Age is a major risk factor for PCa and alterations in DNA methylation occur with aging.⁴⁵ We and others have proposed that age-induced alterations in DNA methylation contribute to PCa development.⁴⁶ The heterogeneous and multifocal nature of PCa poses risks as biopsy tissue may underrepresent the clinical aggressiveness of other tumor foci in the prostate. It is hoped that the molecular analysis of biofluids (blood, urine or other body fluids) may bypass this problem by representing multiple lesions. Techniques for the analysis of methylation are well-suited to high throughput analyses needed for a commercial test and DNA is a relatively stable biomarker source.

In the future, refining epigenetic biomarkers will help resolve common clinical dilemmas among clinicians: when, who, and how patients with PCa should be treated. An improved understanding of epigenetic alterations in PCa will not only provide new and better

biomarker candidates, but have the potential to direct new therapeutic strategies given the reversibility of these processes.

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Key points

Widespread changes in epigenetics underlie prostate cancer behavior. The most broadly investigated areas in biomarker development include DNA methylation and histone post-translational modifications.

High-throughput approaches are leading the discovery of a new wave of markers that detect as well as predict the aggressiveness of prostate cancer. Future assays will likely involve gene panels rather than single genes.

Epigenetic assays are applicable to tissue, as well as biofluids including urine, serum and semen.

To date, the most advanced product has been the Confirm MDx assay, which is CLIA not FDA approved, and being marked to improve the detection of prostate cancer from non-tumor biopsy material.