Chapter 8
Recent Updates on Epigenetic Biomarkers for Prostate Cancer

Karen Chiam, Tanya Kate Day, and Tina Bianco-Miotto

Abstract Epigenetics refers to DNA methylation, histone modifications and microRNAs and these epigenetic modifications are extensively investigated as potential biomarkers for cancer. Characterizing genome wide epigenetic changes involved in prostate cancer development and progression will not only identify potential novel therapeutic targets, since some epigenetic modifications are reversible, but also highlight which epigenetic changes can be used as prostate cancer biomarkers. Epigenetic changes are relatively stable and easy to measure in peripheral samples like blood and urine, further highlighting their importance as powerful tools for assessing patient diagnosis and prognosis. In this review, we outline how epigenetic biomarkers have been used for diagnosis, prognosis and for monitoring therapeutic response in prostate cancer. We also review how epigenetic biomarkers may be more sensitive and specific than current prostate cancer serum markers and the possibility that combining different epigenetic modifications may further enhance the diagnostic and prognostic ability of these epigenetic biomarkers. As epigenome wide studies continue to be performed in larger patient cohorts, we will soon identify

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the epigenetic modifications involved in prostate tumorigenesis with the resultant identification of new therapeutic targets and robust prostate cancer biomarkers.

Keywords DNA methylation • Histone modifications • MicroRNA • Diagnostic biomarkers • Prostate cancer

8.1 Introduction

Prostate cancer is one of the most commonly diagnosed cancers in men of developed Western countries. Globally, it is the second most commonly diagnosed and sixth leading cause of cancer death in men [1]. Risk factors for developing prostate cancer include family history, race, obesity, diet and other environmental factors. However, age is the best known risk factor for prostate cancer, with 80 % of men developing the disease by 80 years of age [2]. Hence, globally, prostate cancer is a major health and economic burden in the aging population.

When prostate cancer is diagnosed at an early organ-confined stage it is potentially curable by radical prostatectomy, which is the surgical removal of the prostate gland. Radiotherapy is another treatment option that is administered either alone or in combination with radical prostatectomy. However, after initial treatment with curative intent, it has been estimated that approximately 30 % of patients will subsequently relapse with metastatic disease. It is this metastatic prostate cancer, present either at the time of diagnosis or developing after failure of primary therapy, which is the primary cause of mortality from prostate cancer. In the 1940s, it was discovered that prostate cancer is dependent on the male sex hormones androgens for growth and survival. Based on this discovery, therapies targeting androgen production and its mediator, the androgen receptor (AR), were developed. These therapies have been the mainstay for treating patients diagnosed with metastatic disease or progressive disease. Anti-androgens that block the functional action of AR are called hormonal or androgen ablation therapy [3]. Prostate tumors treated with hormonal therapy initially regress in most men, but tumors then become unresponsive to these therapies and progress to the castrate-resistant state after a median time of 18–24 months [4]. In its castrate-resistant state [3], all treatment options for prostate cancer are palliative in nature and have limited benefits in improving patient survival.

8.2 PSA: The Controversial Prostate Cancer Biomarker

The limitations of current prostate cancer treatments highlight a major clinical problem, which is to select the optimal treatment strategy for individual patients at the time of diagnosis. The heterogeneous nature of prostate cancer means that they can be either indolent or aggressive. Patients presenting with the same clinical disease

stage may ultimately have very different outcomes. Since the majority of prostate cancer occurs in elderly men, patients with indolent disease are more likely to die with prostate cancer rather than from the disease. This group of men could avoid treatment altogether, and escape complications and side effects commonly associated with treatment. In contrast, men with aggressive prostate cancer are more likely to benefit and should receive aggressive therapeutic intervention. The ability to discriminate between aggressive and indolent disease at the time of diagnosis will have an extremely positive impact on the quality of life and actual treatment benefits to prostate cancer patients.

Unfortunately, current investigational procedures and prognostic nomograms, which are based on clinical features of the disease, do not accurately identify at diagnosis, patients with disease that is likely to become aggressive and life threatening. Identification of patterns of changes in gene expression, or variations in gene structure or sequence early in prostate tumorigenesis provides an opportunity to define at an early disease stage those cancers that are likely to become life-threatening. The current standard biomarker for detecting and predicting prostate cancer progression is the measurement of serum prostate specific antigen (PSA) level. However, there is constant debate in regards to the efficacy of PSA in the clinical setting for the following reasons [5, 6]:

1. There is no specific cut-off serum PSA level that defines if a patient has prostate cancer. Generally, a high serum PSA level indicates the presence of prostate cancer cells, although it has been shown that a proportion of men with high serum PSA levels do not have prostate cancer [7]. Conversely, approximately 22 % of men with prostate cancer have low serum PSA levels [8]. The false positive and false negative results associated with the serum PSA test means that some men without prostate cancer will unnecessarily undergo an invasive needle biopsy procedure, while in some men their prostate cancer will remain undetected.
2. The serum PSA biomarker is not prostate cancer specific. Increased serum PSA level may be indicative of other prostatic diseases, such as benign prostatic hyperplasia (BPH) and prostatitis. BPH is common in elderly men, with a 75–90 % incidence in men by the age of 80 years, [9, 10] and is therefore a confounding factor in interpreting serum PSA results for some men.
3. Serum PSA levels do not distinguish between indolent and aggressive disease at the time of diagnosis. This is coupled with the fact that the implementation of screening protocols which promotes regular testing of serum PSA levels has resulted in the detection of a high proportion of low stage and low grade prostate cancers. Together, this makes clinical decisions about whether or how to treat the prostate cancer difficult. This is a particular issue for older men with a life expectancy of less than 10–15 years, or men with other medical conditions, who may die from other causes before the prostate cancer becomes a problem for them clinically. In these cases, men and their treating clinicians have to decide between treatment or watchful waiting, which is not an easy decision in the absence of accurate clinical information regarding the likelihood of prostate cancer progression in these men.

4. Monitoring changes in PSA levels can assist clinicians to gauge treatment efficacy. However, this requires ongoing monitoring over a period of time, resulting in a time lag before clinicians can identify if the treatment is working. This means that men may have to receive aggressive treatment such as chemotherapy, with associated unpleasant side-effects, for a prolonged time period before its efficacy can be determined.

Two large clinical trials have recently investigated the effect of serum PSA screening on prostate cancer patients survival in the US (n = 76,693 men) and Europe (n = 182,000 men), with contradicting results reported [5, 6]. There was no significant difference in prostate cancer mortality between patients who underwent annual PSA screening test compared to the control group in the US study, whereas the European study reported a 20 % decrease in prostate cancer mortality associated with PSA screening. A meta-analysis of six randomized controlled trials, including the US and European trials mentioned above, does not support the usefulness of PSA screening on reducing prostate cancer mortality [11].

An ongoing major focus of the prostate cancer research community is to identify better biomarkers or improve current PSA measurements for prostate cancer, yet few biomarkers investigated so far improve upon the diagnostic and prognostic value of serum PSA [12–14]. Two potential prostate cancer biomarkers which warrant further investigation are urinary PCA3 (FDA-approved in February 2012) and urinary TMPRSS2-ERG, both of which aid in the detection of prostate cancer when combined with serum PSA. PCA3 may be helpful in cases where men present with abnormal digital rectal examination (DRE) and/or high serum PSA levels coupled with a negative biopsy. In these cases, a low or negative PCA3 score can be used to determine if a repeat biopsy is necessary or may be avoided [15]. TMPRSS2-ERG gene fusion is the most common gene fusion in prostate cancer, occurring in around 50 % of all cancers [16]. This gene fusion is a potential diagnostic biomarker for prostate cancer detection. A combined measurement of PCA3 and TMPRSS2-ERG may serve as a biomarker of prostate cancer, and is currently under further investigation and validation [17, 18]. Despite these advances, the interpretation of both PCA3 and TMPRSS2-ERG diagnostic biomarkers are still dependent on serum PSA levels. In addition, they are not sufficiently characterized, sensitive or specific to enable their use to predict disease progression or treatment response in the clinical setting.

While the search for prostate cancer biomarkers continues, given the relatively slow progress in this field to date, a new approach is required. There is now rapidly accumulating evidence showing the important contribution of epigenetic modifications to all stages of prostate tumorigenesis [19–22], which may be utilized as novel candidate biomarkers for prostate cancer. In this chapter, we will discuss published studies that have identified and investigated candidate prostate cancer epigenetic biomarkers, as well as the challenges faced in this endeavor and the latest advancements in this research field.

8.3 Epigenetic Biomarkers for Prostate Cancer

Epigenetic alterations are common in prostate cancer and are associated with all stages of tumorigenesis, from initiation to progression of the disease [19–22]. While the exact mechanisms of how these epigenetic changes arise in prostate cancer have not been clearly delineated, they occur at a much higher frequency than mutations, and occur commonly in premalignant stages of the disease [23]. These features make epigenetic modifications attractive biomarkers for diagnosis, prognosis and treatment response (Fig. 8.1).

8.3.1 DNA Methylation Based Biomarkers

DNA methylation is a highly stable epigenetic modification involving the addition of a methyl group to the 5′ carbon of a cytosine residue. This occurs predominantly within cytosine-guanine dinucleotide residues. Since DNA is so stable, analysis is technically relatively simple. Added to this, DNA is found in bodily fluids such as blood, urine and saliva. Medical tests on body fluids are non-invasive and therefore ideal in a clinical setting. All of these factors make DNA methylation biomarkers for prostate cancer attractive for further investigation and discussion.

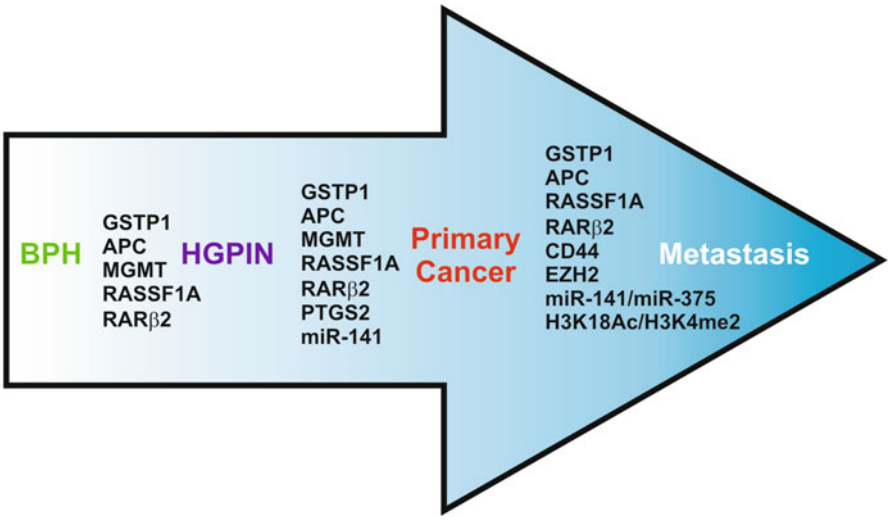

Fig. 8.1 Epigenetic modifications as diagnostic, prognostic and treatment response biomarkers in prostate cancer. Epigenetic alterations that have previously been tested as biomarkers in prostate cancer and cited in the text. BPH refers to benign prostatic hyperplasia and HGPIN refers to high grade prostatic intraepithelial neoplasia

In cancer, global hypomethylation occurs in conjunction with gene-specific promoter hypermethylation [24]. Global hypomethylation is a loss in total genomic DNA methylation, which is linked to activation of proto-oncogenes and chromosomal instability [25, 26]. In prostate cancer, global hypomethylation is associated with metastatic disease [27–30]. An immunohistochemical study performed on human prostate tumor tissues demonstrated a significant decrease in the global 5-methylcytosine levels in patients with recurrent prostate cancer compared to patients without recurrence [28]. Repetitive DNA sequences dispersed in the genome such as retrotransposon elements (i.e. *LINE-1* and *Alu* repeats), which are usually methylated in normal tissues, have been shown to be hypomethylated in prostate cancer [29, 30]. A quantitative methylation-specific PCR (QMSP) study found hypomethylation of *LINE-1* and *Alu* repeats in human prostate adenocarcinoma tissues compared to BPH, and the levels of DNA methylation at these repeat elements correlated with PSA levels and tumor stage [31]. These studies underline the frequency of global methylation changes in prostate cancer. However, none of the above studies investigated if global DNA methylation levels can be used to detect or predict prostate cancer progression. It is only recently that a study has attempted to explore the potential of 5-methylcytosine level to predict survival of patients with prostate cancer [32]. Although a significant decrease in 5-methylcytosine level was observed in the prostate tumors compared to the adjacent normal tissues, there was no association between global DNA methylation levels and patient survival [32].

Up until now, most DNA methylation studies in prostate and other cancers have focused on gene-specific hypermethylation. Gene-specific hypermethylation is an increase in DNA methylation of promoter regions of individual genes. This has been associated with inactivation of genes involved in many cellular functions such as DNA repair, cell-cycle regulation, apoptosis and tumor-suppression [33, 34]. To date, at least 66 genes with promoter hypermethylation have been identified in prostate cancer and is the subject of multiple reviews [20, 21, 35–37]. However, the most frequently occurring and well-studied epigenetic biomarker for prostate cancer is DNA hypermethylation of the glutathione-S-transferase P1 (*GSTP1*) gene promoter.

8.3.1.1 GSTP1 as an Epigenetic Biomarker

GSTP1 encodes an enzyme which is essential for cellular detoxification and protection of DNA from oxidants and electrophilic metabolites [8]. *GSTP1* DNA methylation is an attractive potential epigenetic biomarker for prostate cancer for the following reasons:

1. *GSTP1* DNA promoter methylation is highly specific for prostate cancer (>90 %) compared to serum PSA (~20 %) [38].
2. DNA methylation levels of the *GSTP1* promoter can differentiate prostate cancer from prostatic diseases including BPH and high grade prostatic intraepithelial neoplasia (HGPIN) [39, 40] (Fig. 8.1).

3. *GSTP1* promoter DNA methylation is associated with prostate cancer progression, and disease recurrence after primary therapy [41–43] (Fig. 8.1).
4. *GSTP1* promoter methylation is easily measured in body fluids such as serum, plasma and urine.

There are several published reviews [8, 20, 38, 44–47] highlighting the significance of *GSTP1* hypermethylation as an epigenetic biomarker in prostate cancer. These reviews discussed the techniques and samples (e.g. serum, urine) currently used for analysis and should be referred to for a more detailed insight in this area. Here, we will discuss the features required to further develop *GSTP1* DNA methylation as a robust prostate cancer epigenetic biomarker with utility in the clinic.

Firstly, although *GSTP1* is highly specific for prostate cancer, and more specific than serum PSA, *GSTP1* hypermethlyation does occur in other cancer types. Thus, a key research effort is to enhance the specificity of *GSTP1* as a diagnostic prostate cancer biomarker. One way to achieve this is to measure *GSTP1* DNA methylation in conjunction with a panel of genes with aberrant methylation in prostate cancer [41–43, 48]. The DNA methylation status of a 4-gene panel (*GSTP1*, *RASSF1A*, *RARβ2* and *APC*) has been shown to discriminate prostate cancer patients (n = 95) from age-matched controls (n = 38) with 86 % sensitivity and 89 % specificity [49] (Fig. 8.1). Of particular note, the DNA methylation status was assessed from urine sediments, making this a non-invasive test appropriate for clinical use. Another study demonstrated increased specificity (83–100 %) and sensitivity (94–98 %) when *GSTP1* methylation was combined with *APC* methylation to discriminate between BPH, HGPIN and prostate adenocarcinoma [41] (Fig. 8.1). Using a similar approach, a multi-center study investigated the use of a 3-gene panel (*GSTP1*, *RARβ2* and *APC*) as a diagnostic marker for prostate cancer [50]. The DNA methylation levels of these three genes were assessed by QMSP in the urine samples collected from 337 subjects (178 men with prostate cancer) post DRE and before needle biopsy. The 3-gene panel exhibited an improved accuracy (AUC of 0.57–0.71) compared to serum PSA (AUC of 0.52–0.56), in the detection of prostate cancer [50]. To confirm these findings, the authors performed a similar study in a larger cohort of 704 subjects (320 men with prostate cancer) and demonstrated again that the 3-gene panel (AUC of 0.73) outperformed all other risk factors (i.e. age, serum PSA levels, DRE and family history) (AUC of 0.52–0.66) [51]. These studies demonstrated that as part of a multi-gene biomarker panel, *GSTP1* methylation has great promise as a diagnostic biomarker for prostate cancer diagnosis.

GSTP1 methylation also has potential to act as a prostate cancer prognostic biomarker. The detection of *GSTP1* hypermethylation in patient serum is associated with a 4.4-fold increased risk of biochemical recurrence, measured by PSA relapse [52]. The DNA methylation levels of a 4-gene panel consisting of *GSTP1*, *RASSF1A*, *APC* and *RARβ2* were measured in blood samples from men with prostate cancer, and showed significant association with the risk of biochemical recurrence, although the individual contribution of each gene to this association was not analyzed [53]. In contrast, two other studies have found no correlation between *GSTP1* hypermethylation and biochemical recurrence of prostate cancer [54, 55]; and one study found

that *GSTP1* hypermethylation in human prostate tissue was associated with a decreased risk of biochemical recurrence [56]. The discrepancies among the different studies may be due to differences in characteristics of patient cohorts, methods for DNA methylation analysis and tissue type sampled.

In order to develop an epigenetic biomarker, whether it is an individual gene or a panel of genes, the sample type, timing of collection, and analysis method all require optimization to achieve the greatest sensitivity and specificity. *GSTP1* methylation has been assessed in tissue samples (biopsy or surgically-excised tumor fragments) and also in bodily fluids, including blood, serum, plasma and urine. Assessment using bodily fluids is clearly less invasive and a more desirable option for a biomarker. Wu et al. [38] performed a meta-analysis of over 20 studies, comparing the sensitivity and specificity of *GSTP1* DNA methylation in bodily fluids as a prostate cancer biomarker. *GSTP1* specificity was not influenced by analysis method or sample type; however the sensitivity of *GSTP1* as a biomarker was lower in whole blood and in samples that were collected after treatment, compared to other fluid-based sample types (i.e. plasma and serum). This suggests that for optimal biomarker assessment, samples should be collected prior to treatment, where possible. Obviously, however, if samples are being collected to monitor or measure treatment response, samples need to be collected before and after treatment. Future studies should carefully consider the timing and type of sample collected.

To date, most studies have focused on developing DNA methylation-based markers, including *GSTP1*, as diagnostic and prognostic biomarkers for prostate cancer, and not as a biomarker of treatment response. In principle, in pre-clinical studies and in clinical drug trials, analysis of gene promoter hypermethylation can be employed to assess the efficacy of epigenetic therapeutic agents such as the demethylation agent 5-aza-2′-deoxycytidine (5-aza). Recently, *GSTP1* promoter DNA methylation and re-expression was assessed in human prostate cancer cells after treatment with the demethylating agent 5-aza [57]. *GSTP1* demethylation alone was associated with suppression of cellular proliferation; whereas *GSTP1* demethylation coupled with protein re-expression occurred concomitantly with suppression of proliferation and induction of cell death. Based on this, *GSTP1* presents an attractive target for further testing as a marker of epigenetic therapy response in future clinical trials. In addition to epigenetic therapy response, *GSTP1* has not been investigated as a marker of response to current treatments for prostate cancer, including hormonal therapy and/or chemotherapy. An epigenetic biomarker of treatment response that improves upon the current practice of monitoring serum PSA levels over time, would be of great benefit to patients and their clinicians, by giving information about treatment efficacy earlier in the treatment cycle. An interesting study by Horvath et al. [58] examined methylated *GSTP1* in the plasma of human prostate cancer patients with castrate-resistant disease to investigate if *GSTP1* was predictive of chemotherapy response and survival in these patients. Methylated *GSTP1* levels were measured before and after the first chemotherapy cycle using quantitative methylation-specific head-loop PCR. Patients with decreased methylated *GSTP1* levels after the first chemotherapy cycle were more likely to present a >50 % decrease in PSA levels prior to the fourth chemotherapy cycle (n = 40). Patients with detectable methylated

GSTP1 had a poorer overall survival (23 % survival rate) compared to patients with undetectable methylated *GSTP1* (71 % survival rate) (n = 75), supporting the use of DNA methylation of *GSTP1* as a potential chemotherapy efficacy biomarker for prostate cancer.

8.3.2 Histone Modifications as Biomarkers in Prostate Cancer

Specific histone modifications such as H4K16Ac and H4K20Me3 have been shown to be prognostic in several cancers [59–68]. However, there have only been four studies investigating histone modifications as prognostic markers in prostate cancer [61, 64, 66, 67]. Furthermore, in contrast to the DNA methylation-based biomarkers that have been tested as both diagnostic and prognostic tools for prostate cancer, no study has investigated whether specific histone modifications may be used as diagnostic biomarkers for prostate cancer. The potential of histone modifications as indicators of treatment response in prostate cancer has not been explored and is generally under-studied in all other cancers too, with only two studies (pancreatic and nasopharyngeal cancers) reported in the literature so far [69, 70].

The notion of histone modifications as prognostic biomarkers in cancers was first established in a prostate cancer cohort [61]. Global levels of histone modifications (H3K9Ac, H3K18Ac, H4K12Ac, H3K4Me2 and H4R3Me2) were examined by immunohistochemistry in human primary prostate tumor tissues [61]. With the exception of H3K9Ac, there was a correlation between global levels of all histone modifications and prostate tumor stage [61]. Importantly, the authors demonstrated that combining H3K18Ac and H3K4Me2 predicted tumor recurrence in low grade prostate cancer [61]. A subsequent follow-up study with a larger prostate cancer cohort was able to demonstrate that levels of H3K18Ac and H3K4Me2 were independent predictors of prostate cancer progression regardless of tumor grade [67]. Another histone modification identified to be critical in cancers is H3K27Me3. Loss of H3K27Me3 is common in many cancers and is associated with a poor prognosis [60, 71]. The epigenetic enzyme *EZH2,* which is responsible for H3K27 methylation, is frequently altered during prostate cancer progression and has been shown to be predictive of prostate cancer disease progression [72–78].

The inconsistency and limited studies on histone modifications as cancer biomarkers may be attributed to the lack of technology and methods suitable for the analysis of histone modifications. The most common method used to analyze global expression of histone modifications is immunohistochemistry, which has a relatively low level of sensitivity compared to methods used for DNA methylation analysis. Many experimental factors can also contribute to variations in immunohistochemistry. For example, different antigen-retrieval methods and antibody affinities may affect the immunostaining pattern for a particular histone modification. Most importantly, techniques that can allow accurate measurement of specific histone modifications in body fluids have not been explored, making them less attractive as diagnostic and prognostic biomarkers. The assessment of specific histone modifications in

body fluids is possible following extraction of DNA from serum, plasma or circulating DNA via methods such as ELISA [79, 80]. However, only a single study has utilized ELISA to demonstrate that H3K27Me3 was significantly decreased in metastatic prostate cancer (n = 28) compared to localized disease (n = 33) with an AUC of 0.68 [81]. A recent interesting study investigating the effects of occupational exposure to Nickel on global levels of specific histone modifications (H3K4Me3, H3K9Ac, H3K9Me2) in individuals, has also utilized a similar ELISA approach and found that histone modifications in human peripheral blood mononuclear cells are stable over a period of time [82]. The outcomes of these studies further support the notion of a non-invasive and stable histone biomarker for prostate cancer detection, prognosis and indicators of treatment response may soon be possible.

8.3.3 miRNAs as Biomarkers in Prostate Cancer

An upcoming area of biomarker research in prostate cancer is microRNAs (miRNAs). Studies have shown that miRNAs are of diagnostic and prognostic value for prostate cancer and may even be superior over DNA methylation and histone modifications as biomarkers. Examples of the desirable traits of miRNAs as biomarkers are: they are present and assessable in body fluids (i.e. blood and serum), they are highly stable and have been shown to be tissue- and tumor-specific [83, 84]. Unraveling miRNAs critical in prostate tumorigenesis will subsequently lead to the discovery of novel miRNA-targeted genes and biological pathways implicated in the disease.

Several miRNAs have been identified to be frequently altered in prostate cancer and discussed in reviews [85–87] and in Chap. 3. A collective of studies have shown that distinct miRNA expression profiles can differentiate between non-malignant and prostate tumors, providing evidence that they can be used as diagnostic and prognostic tools [88–95]. For instance, a study by Schaefer et al. [88] undertook a miRNA microarray analysis followed by RT-PCR validation and identified a miRNA expression profile (n = 15 miRNAs) distinct between normal and prostate tumor tissues (n = 76) with an accuracy of 82 %. Of the 15 miRNAs, several were significantly associated with Gleason score (miR-31, miR-96 and miR-205) and tumor stage (miR-125b, miR-205, miR-222) in a second independent prostate cancer cohort (n = 79). High expression of a single miRNA, miR-96, was shown to be associated with increased risk of prostate cancer biochemical recurrence. In another recent microarray study, a miRNA expression profile consisting of 22 miRNAs was able to distinguish between normal and tumor prostate tissues at high prediction rates (91 and 100 % respectively) [89]. In addition, the authors modeled two miRNA expression profiles and investigated them as diagnostic and prognostic biomarkers in the patient cohort used by Schaefer et al. [96]. The modeled diagnostic panel of miRNAs (n = 54) displayed an improved AUC of 0.949 in comparison to that of Schaefer et al. [96]. Most importantly, a separate biomarker panel of prognostic miRNAs (n = 25) displayed an AUC of 0.991 and outperformed Gleason score, pathological stage and serum PSA level in predicting prostate cancer progression [89].

Brase and colleagues [90] generated a profile of the expression of circulating miRNAs (n = 667) in the serum of 21 prostate cancer patients by Taqman miRNA microarray analysis. Further validation of the top five most significantly overexpressed miRNAs (miR-375, miR-9*, miR-141, miR-200b and miR-516-3p) in patients with metastatic compared to localized disease was performed in a separate prostate cancer cohort (n = 45). In this cohort, miR-375, miR-141 and miR-200b were associated with pathological stage and Gleason score. This observation was confirmed in a final validation cohort (n = 71), demonstrating that high expression of miR-375 and miR-141 were significantly associated with pathological stage and Gleason score. The importance of miR-141 and miR-375 as prostate cancer biomarkers was again highlighted by Selth et al. [97], who identified serum miR-141, miR-375, miR-298 and miR-346 levels to be significantly altered in a mouse model of prostate cancer (TRAMP) and in patients (n = 25) with biochemical relapse. High tumor expressions of miR-141 and miR-375 were both significantly associated with increased risk of biochemical recurrence, and miR-375 (HR = 5.70) remained an independent predictor of disease recurrence in multivariate analysis.

8.4 Improvements in Technology and Recent Development of Epigenetic Biomarkers in Prostate Cancer

Identifying methylated DNA requires pre-treatment of DNA, followed by downstream analysis. Historically, the downstream analysis techniques have lent themselves to small scale studies, such as studies of the methylation status of a single gene, or a limited number of candidate genes. Recent technological advances in this area have led to the development of a number of whole-genome methylation techniques, many of which are now broadly accessible and affordable. Coupled with developments in information technology, data from whole-genome epigenetic studies can be integrated with other data sources, opening new doors for the study of epigenetics. In this section, we will discuss some new techniques and concepts in epigenetic study, some of the latest genome-wide studies in prostate cancer, and new epigenetic marks that we consider are likely to make an important contribution to the development of epigenetic biomarkers in clinical prostate cancer.

8.4.1 Genome Wide DNA Methylation: Distinct Profiles and Association with Prostate Cancer Progression

To date, there have been only nine published studies in prostate cancer [98–106] which have utilized at least two independent prostate cancer cell lines and performed an unbiased genome wide analysis of DNA methylation.

One advantage of genome wide methylation analysis is that it is an unbiased technique which can be used to identify methylation of genes or marker DNA regions with potential to act as epigenetic biomarkers of clinical prostate cancer.

Kim and colleagues [103] integrated genome wide DNA methylation results with gene expression, and identified three genes (*PPP1R14C*, *EFEMP1*, *ISL1*) with concordant methylation and expression changes in prostate cancer cells *in vitro*, compared with non-malignant cultured prostate cells. These potential epigenetic biomarkers were validated in clinical samples. *EFEMP1* promoter DNA methylation was the optimal marker to differentiate prostate cancer from BPH, (sensitivity = 95.3 %, specificity = 86.6 %), and this occurred in concert with a reduction in *EFEMP1* gene expression in cancer [103].

Friedlander and colleagues assessed genome wide chromosome copy number, gene expression and DNA methylation changes in metastatic castrate-resistant prostate cancer (CRPC), compared with primary cancer and benign prostate. In this study, 16 genes had concurrent methylation and copy loss in ≥66 % of samples [100], but further validation of these genes as biomarkers of progression was not part of this study. The comprehensive design of this study enabled the authors to demonstrate that DNA methylation changes (10.5 %) occur more commonly than copy number alteration (2.1 %) in CRPC [100]. This observation reinforces the importance of epigenetic biomarkers of prostate cancer, and how they may improve upon PSA serum measurement currently used in clinical practice.

Other genome wide DNA methylation studies have adopted a slightly different approach, and have identified panels of differentially methylated CpGs associated with prostate cancer progression or recurrence. In a clinical cohort, Kobayashi et al. [104] identified a panel of 69 CpGs which were associated with time to biochemical recurrence. These CpGs were located in the promoters of both novel and known cancer-related genes. In the same study, Gleason grade could not be distinguished by DNA methylation profiling. Similarly, Mahapatra et al. [106] analyzed the DNA methylation status of gene promoters and identified panels of genes which were predictive of different types of prostate cancer. A panel of 75 genes could successfully differentiate recurrence from no recurrence, 68 genes could differentiate between systemic recurrence and local recurrence, and 16 genes could differentiate clinical recurrence from biochemical recurrence. A subset of the genes for which promoter DNA methylation was predictive of different types or stages of prostate cancer were validated in an independent clinical cohort. In all cases, this supported the genome wide DNA methylation results, providing further evidence that not only are genome wide techniques highly informative in terms of how many CpGs can be assessed, but they are also accurate and can differentiate between different clinical outcomes or disease stages.

Despite the increasing volume and complexity of data generated, comparisons between studies remain a critical step in selecting biomarkers worthy of further validation and investigation. We sought to determine the degree of similarity between the nine genome wide studies reported to date (Table 8.1). Similarities in genes and gene families identified by genome wide DNA methylation analysis in prostate cancer were assessed. This analysis was somewhat limited by differences in methods, statistical tests used, how the data was presented and made available, clinical versus cell line cohorts, and if the DNA methylation data was combined with gene expression and/or copy number data. Given the multiple sources of technical and biological variation, it was surprising to identify substantial overlap between different

Table 8.1 Genes identified as commonly methylated in prostate cancer by genome wide methodologies

Gene	Cell lines: PCa vs NM	Tumor Vs NM	Progression - Gleason grade	Recurrence
HOXC11	[101]	[106]	[105]	
HOXD3				
HOXD4				
HOXD9				
IRX1				
LBX1				
LHX9				[106]
MNX1				
NKX2		[98]		
SIX6				[106]
VAX1				
AOX1		[104, 106]		
APC	[101, 102]			
BCL2				
C20orf103	[101]		[105]	
CACNA1G	[101, 102]			
CD44				
CDKN2A		[105]		
CYBA		[104, 106]		
ELF4				
FLT4				
GAS6				
GP5	[101]		[105]	
GRASP		[104, 106]		
GRM1	[101]		[105]	
GSTP1	[101, 102]	[104, 106]		
HIF3A				
LAMB3	[99, 101]			
MOBKL2B		[104, 106]		
NEUROG1	[101]			[106]
PYCARD		[104]		
RARB		[104, 106]		
RASSF1	[101, 102]			
RHCG		[104, 106]		
RND2				
RUNX3	[101, 102]	[105]		[106]
SHH	[101]		[105]	
SPATA6		[104, 106]		
SSTR1	[101]		[105]	
TCF7L1				
TFAP2A				
TNFRSF10D				[106]
TPM4		[104, 106]		
WT1	[101, 102]		[105]	
ZNF154		[104, 106]		

studies (Table 8.1). Of note, eight out of nine studies identified genes or gene families overlapping with another study. Only a single study, which had very stringent gene selection criteria and only identified three genes [103], did not have any overlap with any other study. Forty-five genes were identified as differentially methylated in two or more published studies (Table 8.1). As we have discussed earlier in this

chapter, *GSTP1* frequently exhibits prostate cancer-specific gene promoter DNA methylation [107]. This was reflected in the genome wide studies which were included in the comparisons conducted (Table 8.1). The homeobox and T-box gene families (*HOXC11*, *HOXD3*, *HOXD4*, *HOXD9*, *IRX1*, *LBX1*, *LHX9*, *MNX1*, *NKX2*, *SIX6*, *VAX1*) were frequently identified as differentially methylated in cancer compared to non-malignant [98, 101, 106], and during prostate cancer progression [105].

Where studies had more similarities, the number of common genes was higher. For example, focusing on studies which assessed DNA methylation profiles in tumor tissue compared to non-malignant prostate; 16/25 genes identified by Mahapatra et al. [106] were also identified by Kobayashi and colleagues [104]. Similarly, there were many common genes in studies using cell line material (Table 8.1). The degree of similarity also relates to the disease state, with less overlap identified between studies of prostate cancer progression/recurrence than for cell line or cancer versus non-malignant. Taken together, we propose that a general principle is that the degree of variation in DNA methylation between samples is larger than any differences introduced by genome-wide DNA methylation analysis techniques. Therefore, these techniques can provide a reliable and robust measure of genome wide DNA methylation changes. The outcome of our analysis also suggests that, besides the already known gene, *GSTP1*, the homeobox genes may be strong candidates for further development as epigenetic biomarkers for prostate cancer.

8.4.2 The "new" and "under-studied" Epigenetic Marks

The field of epigenetics is constantly expanding and the discovery of 'new-players' creates opportunities for the development of novel biomarkers in cancers. One example is the recent identification of the DNA modification 5-hydroxymethylcytosine, which is a conversion from 5-methylcytosine [108]. Similar to what has been shown with the global loss of 5-methylcytosine in cancers, an immunohistochemical study demonstrated that 5-hydroxymethylcytosine was decreased in prostate cancer [109]. The assessment of both 5-methylcytosine and 5-hydroxymethylcytosine simultaneously may be a better indication of the global levels of DNA methylation. Two other new DNA modifications converted from 5-methylcytosine have also been recently identified, 5-carboxylcytosine and 5-formylcytosine [110], and these warrant further investigation into their roles in prostate cancer and as potential biomarkers.

The advancement in technology is an important factor in the discovery of new epigenetic players in the field. For instance, one recent interesting discovery resulting from the improved technology is the identification of CpG "shores", which are non-CpG islands located outside promoter regions [111, 112]. The methylation status of CpG "shores" demonstrated tissue-specificity and was altered in colon tumors compared to normal colon tissue [111, 112]. Whether this phenomenon of a distinct methylation profile of CpG "shores" may occur in other cancers such as prostate cancer remains to be investigated.

As mentioned earlier (Sect. 8.3.2), histone modifications are under-studied due to the limiting analysis tools to investigate the expression of specific histone modifications in prostate cancer. Genome wide analysis of histone modifications is now possible with methods such as ChiP-sequencing, which allows genomic profiling of multiple specific histone modifications and identifies their interacting proteins that may play an important role in tumorigenesis. One other new technique is the bisulfite sequencing of chromatin immunoprecipitated DNA (BisChIP-seq), which allows high throughput DNA methylation to be studied in conjunction with a specific histone modification (i.e. H3K27me3) [113]. The BisChIP-seq technique enables investigators for the first time to analyze the interaction of DNA methylation and specific histone modifications on the same DNA region, which may provide a better interpretation of the subsequent gene expression data readout. This novel technique may be utilized to investigate significant DNA and/or gene regions of concurrent DNA methylation coupled with specific histone modification as potential epigenetic biomarkers in prostate cancer.

Another area which requires further investigation is the potential of histone variants as epigenetic biomarkers in prostate cancer. Histone variants such as γH2A.X and H2A.Z are known to be markers of DNA damage and genomic stability. For instance, γH2A.X is overexpressed in many cancer cell lines including prostate cancer [100], which suggests it is a potential epigenetic biomarker of treatment response to radiotherapy or other DNA damage-targeting drugs. A potential γH2A.X biomarker for such treatment response is desirable due to the ability to measure nuclei γH2A.X in peripheral lymphocytes [114]. The global level of H2A.Z assessed by immunohistochemistry has been demonstrated to be an independent predictor of survival in a breast cancer patient cohort (n = 500) [115]. While no study has investigated whether H2A.Z predicts prostate cancer progression, a study has shown overexpression of H2A.Z levels in a prostate cancer xenograft mouse model [116].

8.4.3 Implication of Epigenetic Biomarkers in Therapy

The availability of a good epigenetic biomarker will undoubtedly aid the development of epigenetic therapy for prostate cancer in various ways. Firstly, epigenetic biomarkers can be used in clinical trials as indicators of epigenetic drug efficacy. For instance, *GSTP1* promoter DNA methylation and re-expression may be a suitable biomarker in clinical trials testing DNA methylation inhibitors in prostate cancer [57]. Although there are FDA approved DNA methylation inhibitors (i.e. 5-aza-cytidine, 5-aza-2′-deoxycytidine) currently used for the treatment of hematological malignancies, clinical trials with these DNA methylation inhibitors have not been as successful in solid tumors. The failure of previous clinical trials has been attributed to inappropriate dose regimens, leading to toxicity-related adverse events. Using a frequent low-dose 5-aza-2′-deoxycytidine regimen, it has been shown that the DNA methylation and protein expression status of *GSTP1* was an indicator of DNA methylation inhibitor (5-aza-2′-deoxycytidine and Zebularine) treatment

efficacy in prostate cancer cells [57]. Hence, future clinical trials involving currently available or new DNA methylation inhibitors in prostate cancer should utilize epigenetic biomarkers such as *GSTP1* (alone or in combination with a panel of genes) to track drug efficacy in patients in a timely manner. However, *GSTP1* DNA methylation has recently been shown to be a marker of response to chemotherapy [58].

Secondly, the identification of epigenetic biomarkers that may have functionally important roles in prostate tumorigenesis can also be potential therapeutic targets. For example, the histone methyltransferase enzyme EZH2, and its substrate H3K27 methylation, are aberrantly expressed in prostate cancer and predict prognosis in several studies (Sect. 8.3.2). Hence, relatively new epigenetic drugs targeting histone methyltransferases and histone demethyltransferases may be potential treatments for prostate cancer. In particular, the histone methyltransferase inhibitor DZNep that inhibits EZH2 activity has been shown to reduce prostate cancer cell growth *in vitro* and *in vivo* [117]. This deserves further investigation and validation of its potential therapeutic use in prostate cancer. We also propose that in future studies, the global levels of H3K27 methylation might be a potential biomarker to determine treatment efficacy for histone methylatransferases like DZNep.

8.5 Future Directions

There is compelling evidence that epigenetic biomarkers for the diagnosis and prognosis of prostate cancer are very promising (Fig. 8.1), but currently, there are few clinical trials investigating these biomarkers for such purposes. From a search in the clinicaltrials.gov database, only three clinical trials were found; two trials investigating a panel of hypermethylated genes in urine and serum as an early detection marker (NCT00340717 and NCT01441687) and a single trial aiming to investigate the association of a miRNA expression profile as a prognostic biomarker (NCT01220427). Several reasons may contribute to the impediment of translating prostate cancer epigenetic biomarkers into clinical trials. Firstly, there is a lack of understanding of the biological significance of these candidate epigenetic biomarkers in prostate tumorigenesis. This is coupled with a lack of consistency in experimental designs to test these biomarkers, and until recently, the limitation of technology available for analysis. Additionally, there are other important factors that should be taken into consideration but have often been overlooked in previous studies investigating the use of epigenetic biomarkers in prostate cancer. For example, since epigenetic alterations arise normally during aging, consideration needs to be made to whether the epigenetic biomarker of interest may also undergo an age-related epigenetic change, especially since prostate cancer is an aging-associated disease.

Nevertheless, with the advancement and availability of state of the art technology for global epigenome analyses, as well as the decrease in the cost of these technologies, the critical epigenetic alterations involved in prostate tumorigenesis will be identified. This will then provide a valuable resource for identifying epigenetic biomarkers that can be used as powerful tools for diagnosis, prognosis and therapy response in prostate cancer.

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