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REVIEW

Prostate cancer epigenetic biomarkers: next-generation technologies

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Cancer is caused by a combination of genetic alterations and gross changes to the epigenetic landscape that together result in aberrant cancer gene regulation. Therefore, we need to fully sequence both the cancer genome and the matching cancer epigenomes before we can fully integrate the suite of molecular mechanisms involved in initiation and progression of cancer. A further understanding of epigenetic aberrations has a great potential in the next era of molecular genomic pathology in cancer detection and treatment in all types of cancer, including prostate cancer. In this review, we discuss the most common epigenetic aberrations identified in prostate cancer with the biomarker potential. We also describe the innovative and current epigenomic technologies used for the identification of epigenetic-associated changes in prostate cancer and future translational applications in molecular pathology for cancer detection and prognosis.

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INTRODUCTION

Prostate cancer is the most prevalent cancer and the second leading cause of cancer death in men.[1] When diagnosed early, prostate cancer is potentially curable by surgical removal of the prostate and/or radiotherapy. However, up to 20% of patients will relapse to metastatic disease in 5–10 years. The main therapy for these patients targets androgen production and the androgen receptor, called hormonal or androgen ablation therapy. However, a significant proportion of prostate cancers become unresponsive and progress to a castrate-resistant prostate cancer with limited treatment options.[2] Serum prostate-specific antigen (PSA) screening is the gold standard biomarker for prostate cancer diagnosis and management. However, the PSA test has poor specificity, leading to overdiagnosis and overtreatment of patients with latent cancers that are unlikely to cause significant symptoms or mortality.[3,4] Thus, development of new biomarkers to stratify risk of prostate cancer aggressiveness at the time of screening remains the greatest unmet clinical need in prostate cancer management. Here, we propose that epigenetic-based biomarkers may provide the next future step in prostate cancer diagnosis and prognosis, especially as genome-wide sequencing and analysis approaches for prostate cancer are further implemented.

Epigenetic alterations are a common feature of prostate cancer, and in addition to genetic alterations, they have a critical role in cancer initiation and progression. Epigenetics define the different molecular mechanisms that alter gene function and regulation during development and differentiation without affecting the DNA sequence. Importantly, unlike transient gene regulation, the epigenetic state is inherited to each daughter cell during somatic cell division to ensure faithful cell-type-specific gene expression. There are a number of epigenetic processes identified, each of which can work independently or together to modulate gene regulation and form the 'epigenome'. These primarily include: DNA methylation, posttranslational histone modifications, the incorporation of histone variants and expression of non-coding RNAs, as summarised in Figure 1. Mapping the cancer 'epigenome' to integrate the different layers of epigenetic change that occur 'on top' of the cancer genome will aid in understanding the molecular mechanisms involved in cancer initiation and will potentially provide a new mode of molecular pathology in cancer detection in all types of cancer, including prostate cancer. Therefore, high-throughput genome and protein-based technologies, such as next-generation sequencing (NGS) and immunohistochemistry (IHC), are facilitating the rapid translation of basic molecular research.

In this review, we discuss the most common epigenetic aberrations identified to date in prostate cancer with respect to the biomarker potential (Table 1), as well as summarise the current innovative technologies (Table 2) that are being used to identify new alterations in the cancer epigenome and future translational applications.

EPIGENETIC BIOMARKERS IN PROSTATE CANCER: PRESENT AND FUTURE

DNA methylation as a biomarker in prostate cancer

DNA methylation (5-methylcytosine (5mC)) occurs during normal development in higher organisms, primarily at cytosine residues next to guanine residues (CpG dinucleotides) (Figure 1). CpG sites occur with a low frequency in vertebrates containing only few areas with higher CpG density, termed 'CpG islands'. Low levels of cytosine methylation in non-CpG contexts have also been identified by methylome sequencing in embryonic stem cells, oocytes and brain,[5–8] but it is still unclear if non-CpG methylation has any functional role in normal development and cancer. In contrast, there is a profound alteration of the CpG DNA

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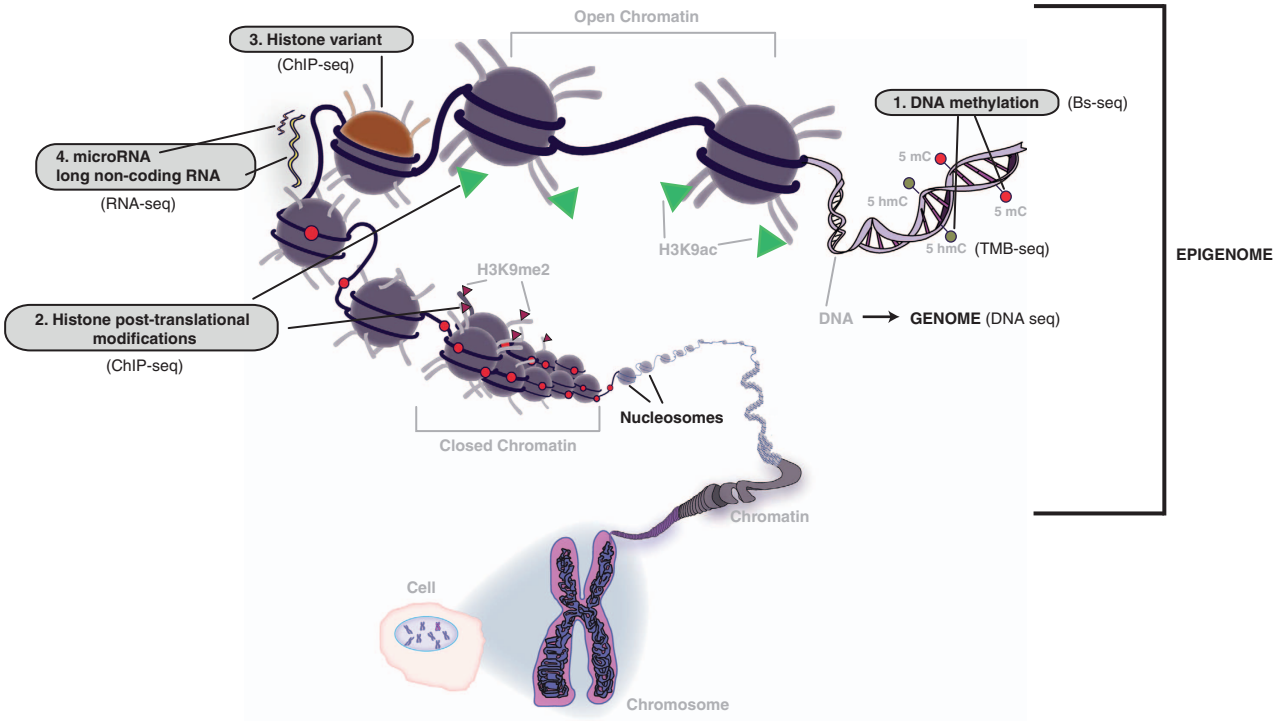

Figure 1. Epigenetic mechanisms. There are four main epigenetic mechanisms processes (highlighted in grey boxes) studied in prostate cancer that require different technology (within parentheses) for their detection. The integration of genomics and epigenomics data would give invaluable knowledge in prostate cancer and lead to the discovery of new biomarkers.

methylation landscape that occurs in the early stages of cancer initiation and continues to change during cancer progression.[9] These alterations are characterised by a global DNA hypomethylation that is thought to promote genome instability and oncogene activation, and local DNA hypermethylation typically at CpG island promoters of tumour suppressor genes associated with gene silencing.

Genomic DNA hypomethylation occurs in both early[10,11] and late stages of prostate cancer[12–14] and has been proposed to be a potential biomarker for early detection and prognosis (Table 1). 5mCpG promoter hypermethylation is the best-characterised epigenetic alteration in prostate cancer.[15] To date, most of the hypermethylation loci currently reported as potential prostate cancer detection and prognosis biomarkers have been assessed by candidate gene studies (summarised in Table 1). Notably, glutatione *S*-transferase pi 1 (*GSTP1*) is hypermethylated in >90% prostate cancer[16] and is a promising epigenetic biomarker for early cancer detection.[17,18] Commercialised assays have been developed for the detection of *GSTP1* methylation with high specificity (86–100%) for prostate cancer; however, the sensitivity is variable depending on the methylation assay design and sample type, urine (19–83%),[19–23] serum or plasma (13–72%).[24,25] To improve prostate cancer sensitivity, multigene promoter methylation testing has been suggested[22,26,27] (Table 1). For example, combining the prostate cancer methylation assay targeting *GSTP1*, *APC* and *RARB2* promoters, the overall predictive power was increased in men with PSA levels 2.0–10.0 ng/ml, and was associated with an increased likelihood of having higher Gleason score in the biopsy.[28]

DNA 5-hydroxymethylation in prostate cancer and its future as biomarker. In addition to 5mC, it is now clear that 5-hydroxymethylcytosine (5hmC) also occurs in mammalian cells (Figure 1). In 2009, TET proteins (*TET1–3*) were shown to mediate the oxidation of 5mC into 5hmC in embryonic stem cells and Purkinje neurons.[29,30] TET-mediated 5mC oxidation is reported to initiate DNA demethylation, both by passive and by active mechanisms in postfertilisation reprogramming.[31,32] 5hmC might also function to modulate the binding of chromatin effectors to change gene expression.[33]

Notably, the global levels of 5hmC have found to be strongly reduced in cancer, including prostate cancer[34–36] and mutations in the *TET2* gene are commonly observed in human myeloid malignancies that also display low levels of 5hmC but high levels of 5mC.[37,38] In prostate cancer, 5hmC and Ki67 presence was mutually exclusive, suggesting that the combined analysis of Ki67 and 5hmC levels could be developed into a biomarker for prostate cancer diagnosis. The involvement of 5hmC in tumour development was further validated in a mouse model of prostate cancer where a gene-independent 5hmC reduction during tumour development was observed.[36] TET1 depletion facilitates cell invasion, tumour growth and cancer metastasis in prostate cancer xenografts,[39] suggesting that low levels of 5hmC may have a future potential as a prognostic biomarker.

Non-coding microRNAs as biomarkers in prostate cancer

MicroRNAs (miRNA) are a class of small non-coding RNAs (18–25 nucleotides) that bind and inhibit target sites in the 3′-untranslated region of specific mRNAs (Figure 1). Each miRNA can potentially bind and inhibit 200 or more different mRNAs simultaneously, and also each mRNA can be targeted by multiple miRNAs. miRNA expression is altered in cancer cells by both genetic and epigenetic mechanisms, potentially affecting both oncogene and tumour suppressor gene regulation.[40]

Deregulation of miRNAs in prostate cancer has been widely related with local invasion[41] and in early stages of the prostate[42,43] (Table 1). miRNAs have been shown to be present and are relatively stable in clinically accessible biofluids such as serum, plasma, urine and saliva, suggesting that miRNAs have the potential as biomarkers using non-invasive assays.[44–46]

Table 1. Epigenetic biomarkers in prostate cancer

Epigenetic mechanism	*Alteration*	*Gene altered*	*Biomarker*	*References*
DNA methylation	Hypomethylation	Global DNA hypomethylation	Prognosis	Brothman *et al.*,[12] Cho *et al.*,[13] Yegnasubramanian *et al.*[14]
			Early detection	Yang *et al.*,[11] Cho *et al.*[10]
		IGF2	Early detection	Jarrard *et al.*,[127]; Bhusari *et al.*,[128]
	Hypermethylation	GSTP1	Early detection	Nakayama *et al.*,[17] Goessl *et al.*,[20] Gonzalgo *et al.*,[21] Hoque *et al.*,[22] Jeronimo *et al.*,[25] Devaney *et al.*,[129]
			Diagnosis	Cairns, *et al.*,[19] Goessl *et al.*,[20,24]
			Prognosis	Gonzalgo *et al.*,[21] Bastian *et al.*,[113]
		GSTP1/APC/MDR1	Diagnosis	Enokida *et al.*[27]
		p16/ARF/MGMT/GSTP1	Early detection	Hoque *et al.*[22]
		GSTP1/APC/RARB2/RASSF1A	Early detection	Roupret *et al.*[23]
		GSTP1/APC/RARB2	Early detection Prognosis	Baden *et al.*[28]
		GSTP1/PTGS2/RPRM/TIG1	Prognosis	Ellinger *et al.*[26]
		APC	Prognosis	Henrique *et al.*[114]
		GSTP1/APC/PTGS2/MDR1	Prognosis	Ellinger *et al.*,[26] Bastian *et al.*,[115] Yegnasubramanian *et al.*[116]
			Diagnosis	Enokida *et al.*[27]
		PTGS2/CD44	Prognosis	Woodson *et al.*[117]
		GPR7/ABHD9/Chr3-EST	Prognosis	Cottrell *et al.*[118]
		PITX2	Prognosis	Banez *et al.*[119]
5-Hydroxymethylcytosine	Loss of 5hmC	Global levels	Diagnosis	Jin *et al.*,[34] Yang *et al.*[36]
miRNAs	Downregulation	miR-16/ miR-31/miR-125b/ miR-145/ miR-149/ miR-181b/ miR-184/ miR-205/ miR-221/ miR-222	Early detection	Schaefer *et al.*[42]
		miR-29a	Early detection	Li *et al.*[43]
	Upregulation	miR-96	Early detection Prognosis	Schaefer *et al.*[42] Schaefer *et al.*[42]
		miR-141	Early detection Prognosis	Mitchell *et al.*[44] Brase *et al.*,[45] Mitchell *et al.*[44]
		miR-182/ miR-183	Early detection	Schaefer *et al.*[42]
		miR-375	Early detection Prognosis	Schaefer *et al.*[42] Schaefer *et al.*,[42] Brase *et al.*[45]
		miR-194	Prognosis	Tong *et al.*[96]
		miR-34c	Prognosis	Hagman *et al.*,[130]
		miR-205	Prognosis	Schaefer *et al.*,[42] Hulf *et al.*[97]
Histone modifications	Downregulation	H3ac/H3K9me2	Early detection	Ellinger *et al.*,[134]
		H4K20me 1, 2 and 3	Prognosis	Fraga *et al.*,[131]; Behbahami *et al.*,[132]
		H3K27me3	Early detection Prognosis	Pellakuru *et al.*[52]
	Upregulation	H3K18Ac/H3K4me2	Prognosis	Bianco-Miotto *et al.*[100] Seligson *et al.*,[133]
		H3K4me1	Prognosis	Ellinger *et al.*,[134]
		H3K27me3	Prognosis	Ellinger *et al.*,[51]
Histone modifier proteins	Upregulation	EZH2	Prognosis	Varambally *et al.*,[57] Laitinen *et al.*[58]
		LSD1	Prognosis	Kahl *et al.*,[135]

Abbreviations: GSTP1, glutatione *S*-transferase pi 1; 5hmC, 5-hydroxymethylcytosine; IGF2, insulin-like growth factor 2; LSD1, lysine-specific demethylase 1; miRNA, microRNA.

For example, the serum levels of miR-141 are able to distinguish patients with prostate cancer from healthy controls[44] and serum miR-141 levels are significantly correlated with more aggressive prostate cancer.[44,45] Long non-coding RNAs are also deregulated in prostate cancer, suggesting their potential use as biomarkers.[47]

Histone modifications and histone chromatin-modification enzymes in prostate cancer: biomarker potential

Posttranslational modifications (for example, acetylation, methylation, phosporylation, sumoylation and ubiquitylation) can occur on the N-terminal histone 'tails' and the combination of histone modifications (known as 'the histone code')[48] confer local

Table 2. Technology tools for cancer epigenetic analysis

	Epigenetic mechanism	*Method*	*References*
Gene by gene analysis methods	DNA methylation	MSRE	Reviewed in Fraga and Esteller[77]
		MSP	
		Pyrosequencing	
		COBRA	
		Ms-SNuPE	
		Bisulphite clonal sequencing	
	miRNAs	RT-qPCR	Leite *et al.*[95]
	Histone modifications/histone modifiers/histone variants	ChIP qPCR	Coolen *et al.*,[55] Bert *et al.*,[56] Valdes-Mora *et al*,[72] Ngollo *et al.*[98]
Microarray-based methods	DNA methylation	MeDIP-chip	Weber *et al.*[120]
		MBD-chip	Robinson *et al.*[87]
		DNA methylation arrays	Yu *et al.*,[83] Irizarry *et al.*[84]
		Infinium methylation arrays	Sandoval *et al.*,[89] Kobayashi *et al.*,.[85] Dedeurwaerder *et al.*[86]
		Sequenom MassARRAY	Coolen *et al.*, [136]
	miRNAs	miRNA microarrays	Schaefer *et al.*,[42] Tong *et al.*[96]
		Low-density Taqman arrays	Brase *et al.*[45]
	Histone modifications/histone modifiers/histone variants	ChIP-chip arrays	Yu *et al.*,[54] Coolen *et al.*,[55] Bert *et al.*,[56] Valdes-Mora *et al.* [72]
		TMAs	Bianco-Miotto *et al.*[100]
NGS-based methods	DNA methylation	BS-seq	Berman *et al.*,[121] Gu *et al.*[122]
		MBD-seq	Serre *et al.*[123]
		M-NGS	Kim *et al.*[110]
		MeDIP-seq	Kim *et al.*[110]
	miRNAs	Small RNA sequencing	Martens-Uzunova *et al.*,[124] Ozsolak and Milos[125]
	Histone modifications/histone modifiers/histone variants	ChIP-seq	Barski *et al.*[126]
Others	5-Hydroxymethylcytosine	IHC	Jin *et al.*,[34] Yang *et al.*[36]
		LC-MS/MS	Jin *et al.*[34]
		Dot blots	Jin *et al.*,[34] Yang *et al.*,[36]
	Histone modifications/histone modifier proteins/histone variants	IHC	Lorincz[99]
		ELISA	Holdenrieder *et al.*,[137] Deligezer *et al.*,[138]

Abbreviations: Bs-seq, whole-genome bisulfite sequencing; chIP, chromatin immunoprecipitation; COBRA, Consolidated Omnibus Budget Reconciliation Act; chlp-chip arrays, chromatin immunoprecipitation followed by tiling arrays; chlp-seq, chromatin immunoprecipitation followed by next generation sequencing; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; LC-MS/MS, liquid chromatography-mass spectrometry; MBD-chip, methyl capture using complex of methyl binding proteins MBD2 followed by tiling arrays; MBD-Seq, methyl capture using complex of methyl binding proteins MBD2 followed by next generation sequencing; MeDIP-chip, methylation DNA immunoprecipitation followed by tiling arrays; MeDIP-seq, methylation DNA immunoprecipitation followed by next generation sequencing; M-NGS, methylPlex-next-generation sequencing; miRNA, microRNA; MSP, methylation-specific PCR; MSRE, methylation-sensitive restriction endonuclease; Ms-SNuPE, methylation-sensitive single-nucleotide primer extension; NGS, next-generation sequencing; RT-qPCR, quantitative reverse transcription; TMA, tissue microarray.

structural changes to the chromatin that affect gene transcription (Figure 1). Histone modification enzymes are responsible for the different posttranslational modifications; these primarily include histone acetyltransferases, deacetylases, methyltransferases and demethylases.[49]

During embryogenesis and development, the histone code forms an epigenetic landscape that determines cell fate decision-making, and fine-tunes gene transcription at specific gene loci.[48] In tumourigenesis, there is deregulation of many of the histone modification enzymes and the homeostasis of the histone code is altered leading to inappropriate gene expression supporting uncontrolled cell proliferation and invasion.[49,50]

Few studies have evaluated the diagnostic, prognostic or predictive role for only global changes of histone modifications in prostate cancer by IHC (Table 1) from primary tumours or the metastasis. For example, global overexpression of the polycomb repressive H3K27me3 mark is found in metastatic prostate tumors and in castrate-resistant prostate cancer compared with localised

prostate cancer and normal prostate tissue.[51] In contrast, an independent study found that global levels of H3K27me3 are reduced in both early during prostatic carcinogenesis and in invasive adenocarcinoma.[52] This contrasting results could be explained owing to the high heterogeneity of the prostate tumours,[53] and suggest that more studies doing microdissection and identifying different cell subpopulation within the tumour are required to establish the potential prognostic value of H3K27me3 levels in prostate cancer.

There are only few studies performed in prostate tissues to investigate the genome-wide reorganization of histone modifications,[54] as most of the genome-wide studies have been carried out in prostate cancer cell lines.[55,56] H3K27Me3 chromatin immunoprecipitation (ChIP)-chip experiments in prostate cancer tissues revealed that a Polycomb repression signature, composed of 14 direct targets of the polycomb group, has been associated with metastatic prostate cancer and a poor clinical outcome.[54]

The most well-characterised histone modification enzyme biomarkers reported in prostate cancer are summarized in Table 1. EZH2 is a histone methyltransferase that forms a Polycomb complex for the H3K27me3. EZH2 is strongly up-regulated in hormone-refractory metastatic prostate cancer and its overexpression is a marker of poor outcome following radical prostatectomy, as assessed by IHC.[57,58] Recent studies have reported that the oncogenic activity of EZH2 in castrate-resistant prostate cancer is independent of its role as a polycomb transcriptional repressor;[52,59] instead, it involves the ability of EZH2 to act as an AR coactivator. Increased expression of various epigenetic AR coactivators TIF-2,[60] p300,[61] CBP,[62] Tip60[63] and recently EZH2[59] are found in castrate-resistant prostate cancer. Taken together, the data suggest that upregulation of epigenetic coactivators during androgen ablation may be relevant to the failure of endocrine therapy in patients with prostate carcinoma.

However, most of these studies have used primary or metastatic tumour samples; thus, it is needed to validate these findings using non-invasive methods. In addition, most of the studies only assess global changes and not localised changes in chromatin remodelling that commonly occurs in domains across the cancer genome.[54–56]

Biomarker potential of histone variants in prostate cancer

Histone variants can replace canonical histones in the nucleosome to produce structural and gene expression alterations distinct from those of the major core histones (Figure 1). They are involved in DNA repair, meiotic recombination, chromosome segregation, transcription initiation and termination, sex chromosome condensation and sperm chromatin packaging.[64] Recently, it has become apparent that some histone variants (H2A.Z, H2A.X, mH2A, CENP-A and H3.3) may also have a critical role in cancer initiation and progression.[65–68]

An oncogenic role for only one histone variant, H2A.Z, has been reported in prostate cancer.[69–72] Acetylated H2A.Z is associated with oncogene activation and the deacetylated form correlates with the silencing of tumour suppressor gene.[72] Further, H2A.Z expression is upregulated in response to androgen treatment and this occurs in part through increased binding of Myc to *H2AFZ* promoter.[71] H2A.Z and its posttranslational modifications, including acetylation[72] and ubiquitylation,[70] have a role in gene transcriptional deregulation in prostate cancer cell lines, but whether this correlates with prostate cancer prognosis requires further investigation.

TECHNOLOGY TOOLS FOR CANCER EPIGENOME ANALYSIS

Epigenetic biomarker detection assays have previously been developed for single gene analyses and these assays (summarised in Table 2) are widely used for the identification of loci-specific epigenetic aberrations in cancer. However, some of these techniques are laborious and restricted to the region(s) of interest, and therefore are not ideal methods to be applied in the clinic for biomarker detection.

Advances in DNA and RNA sequencing have significantly increased our knowledge of the genetic basis of human prostate cancer and the molecular mechanism responsible for disease progression and treatment resistance.[73,74] As a result, biomarker research has shifted to use '-omics' methods for discoveries and profiling tumours for aberrations in DNA, RNA and, more recently, DNA methylation states.[75] Here, we summarise the current and new epigenetic approaches frequently used by researchers to identify novel biomarkers in prostate cancer with special emphasis on novel technologies based on NGS.

Methods for the detection of DNA methylation

There has been a transformation in DNA methylation analysis technology over the past decade for both epigenetic biomarker discovery and cancer detection. The interest in DNA methylation as a cancer biomarker is primarily due to the fact that unlike RNA, DNA is a robust marker and less susceptible to different sample processing techniques and that CpG island promoter methylation, in particular, provides a positive signal for a loss of gene function, such as in the detection of tumour suppressor genes. DNA methylation analysis techniques can be divided into three main approaches: endonuclease digestion, affinity enrichment and bisulphite conversion.[76] Several qualitative and quantitative methods based on a combination of these approaches exist to analyse DNA methylation at specific loci[77] and genome-wide level .[76,78,79] Table 2 provides a summary of the most commonly used methods for DNA methylation analyses to detect candidate biomarkers in prostate cancer.

Gene by gene analysis methods. Methods based on prior bisulphite conversion of the DNA provide the most comprehensive methylation detection for both genome-wide and detection of specific loci. Bisulphite treatment of DNA results in deamination of non-methylated cytosines to uracils, whereas the methylated cytosines remain unaltered.[80] This alteration in the nucleotide sequence allows distinction between 5C methylated and non-methylated sites through polymerase chain reaction (PCR)-based amplification-based protocols. The gold standard method for 5mC detection is genomic clonal bisulphite sequencing, where the region of interest is PCR amplified, cloned and sequenced to provide the exact methylation status of each CpG or non-CpG site at the single-nucleotide level.[81] Traditionally, only 10–20 molecules were sequenced, but with the implementation of high-throughput next-generation technolologies such as MiSeq and Ion Torrent, about 10 000 molecules can be sequenced from each PCR amplicon in a multiplex reaction.[82]

Genome-wide DNA methylation analysis methods. A number of microarray-based technologies used for DNA methylation analyses are now available to study defined genomic entities such as promoters or CpG islands (Table 2). These include arrays containing CG-rich regions of the DNA (DNA methylation arrays);[83,84] whole-genome arrays after bisulphite conversion of the DNA (Infinium 450 K Methylation arrays, Illumina, Inc., San Diego, CA, USA);[85,86] DNA immunoprecipation using specific antibodies against methylated DNA (anti-5meC) followed by tiling arrays (MeDIP-chip) and methyl capture using complex of methyl binding proteins MBD2 (MBD-chip)[87] (Table 2).

The method of choice for DNA methylation biomarker discovery or detection would be influenced by the sensitivity, specificity, accuracy, amount and quality of the starting material, costs, sample throughput, time-consuming and the difficulty of the bioinformatics analysis (see Review for further details[76]). Bock *et al.*[79]

made a comparison study among four popular methods for DNA methylation profile on a genomic scale, to identify the strengths and weaknesses of each method for their utility for biomedical research and biomarker discovery. The four methods used were as follows: MeDIP-seq, MBD-seq, reduced representation bisulphite sequencing[88] and the Infinium HumanMethylation27 assay. Overall, the four methods provided accurate DNA methylation measurements and demonstrated that can be used to detect differentially methylated regions in clinical samples. However, the implementation of the 450K Infinium Methylation Array has significantly improved the coverage issue encountered in the previous version, 27 k Infinium Methylation array,[86,89] and now it has become one of the best choices of the researchers and epigenome projects for biomarker discovery. One example of its application in clinical samples is the recent release of the genome-wide DNA methylation data of 198 cases of prostate cancer by The Cancer Genome Atlas initiative using this technology. Thus, in the next few years the discovery of novel differentially methylated regions in prostate cancer biomarkers will be increased exponentially.

Methods for the detection of 5hmC. Since the discovery of 5hmC, the mammalian genome have fuelled a strong interest in quantifying global levels as well as mapping genomic distribution of 5hmC in various cell types and tissues.

Because standard approaches such as sodium bisulfite sequencing or methylation-sensitive restriction enzymes cannot discriminate 5hmC from 5mC, novel experimental strategies have been developed (reviewed in Wu and Zhang[90] and Booth *et al.*[91]). These strategies include (1) thin-layer chromatography analysis of modified nucleotides, (2) mass spectrometry analysis, (3) modification-specific antibodies, (4) selective chemical labelling of modified cytosine and (5) real-time sequencing. Recently, two modified bisulphite sequencing protocols have been developed to generate for the first time single-base resolution maps of the absolute abundance of 5hmC in mammalian cells.[91,92]

However, the major challenges to analyse human tumour samples are the limitation of starting material combined with the very low levels of 5hmC in the different cell types. 5hmC is estimated to account for 0.59 and 0.03% off all nucleotides in neurons and embryonic stem cells, respectively (5mC constitutes ~1%)[93] and for the other cell types is generally below these ones.[94] For this reason and also for the still limited number of approaches to generate single-base resolution maps of the absolute abundance of 5hmC, the most popular approach to analyse 5hmC in human tissues is to quantify the global levels of 5hmC by using liquid chromatography-mass spectrometry, anti-5hmC antibody-based immuno-dot blots and immunohistochemistry[34] (Table 2). In addition, Yang *et al.*[36] developed a very reliable and quantitative dot-blot assay for determining the change in 5hmC levels using genomic DNA isolated from paraffin-embedded tissue sections, being an ideal assay to be apply for clinical diagnosis.

Methods for the detection of miRNAs

miRNAs can be accurately and easily measured by quantitative reverse transcription (RT-qPCR) (Table 2).[95] Currently, most clinical studies perform microarray analysis to identify miRNAs biomarkers and RT-qPCR to validate specific and relevant miRNAs to finally establish specific miRNA signatures as biomarkers[42,96] (Table 2). Different assays also allow simultaneous quantification by RT-qPCR of a comprehensive panel of individual miRNAs and are widely used to discover novel biomarkers (for example, low-density Taqman arrays).[45,97] Importantly, the identification and validation of miRNAs involved in diagnosis and prognosis will increase exponentially with the data available from The Cancer Genome Atlas for RNA-seq and miRNA-seq.

Methods for the detection of histone modifications, histone chromatin-modification enzymes and histone variants

Table 2 shows the methods commonly used to detect histone modifications, histone chromatin-modification enzymes and histone variants in prostate cancer. ChIP is a routine method to identify binding of histones, modified histones, histone variants or other factors to specific genomic regions. Chromatin is crosslinked and sheared followed by pull down with specific antibodies to the histones and their bound DNA. To examine the change in histone modification of candidate genes in cancer, PCR amplification is performed on the ChIP DNA with primers defining a certain genomic region.[98] ChIP-related technologies can be scaled to genome-wide studies, and ChIP-chip is the most commonly used microarray-based approach to identify histone modifications bound to the DNA and to explore its association with active or inactive chromatin in tumour and normal tissues,[54] although this approach is likely to be soon replaced by ChIP-seq (Table 2).

Global histone modifications and histone modifier proteins are detected using the widely available and low-cost IHC method (Table 2).[99] The high-throughput IHC-based method is the tissue microarray (TMA), which consists of paraffin blocks in which up to 1000 separate tissue cores are assembled in an array manner to allow multiplex histological analysis. TMAs have been used to identify several histone modifications as biomarkers in prostate cancer.[100] However, IHC-based methods are generally a qualitative and subjective.[101] Global levels of histone modifications can now be also detected in cell-free body fluids predominantly as mononucleosomes and oligonucleosomes derived from tumours.[102] Enzyme-linked immunosorbent assays can also quantify circulating histone modifications and nucleosomes (Table 2).

The impact of new technologies in the future of cancer management: NGS

The growing demand for high-throughput genome-wide methods has led to the development of NGS technologies and has initiated a real revolution in genomic analysis.[103] With these technologies, sequencing of the whole human genome is possible within a day and offers exciting possibilities for systematic discoveries and also biomarker detection. NGS enables higher resolution and/or higher genomic coverage at a competitive cost. There are an increasing number of providers on the market for NGS that use different chemistry and methodology for the same goal, these are, 454 GS FLX (Hoffmann-La Roche, Basel, Switzerland), HiSeq2500 (Illumina, Inc.), Ion Torrent Personal Genome Machine and the SOLiD4/5500 (Life Technologies (Thermo Fisher Scientific, Carlsbad, CA, USA)), DNA nanoball sequencing system (Complete Genomics, Mountain View, CA, USA), PacBio RS II (Pacific Biosciences, Menlo Park, CA, USA) and the GridION system (Oxford Nanopore Technologies, Ltd., Oxford, UK). NGS is based on the spatial immobilisation of millions of short DNA fragments followed by a massively parallel sequencing process,[103] providing digital information on DNA fragment. These short sequences are then assembled and aligned to reference genomes using bioinformatics tools. Parallel sequencing of millions of DNA molecules is especially useful for sequencing heterogeneous material, as is the case with cancer tissues. Since the launch of the first NGS platform, NGS companies are doing major efforts to improve the technology to reach the '$1000 genome' and advances have been made, such as improved sequencing chemistry, novel signal detection methodologies, easy sample preparation, multiplexing for a higher sample throughput and reduced cost.[104] Thus, sequencing a cancer genome in a patient will soon be clinically feasible and could be incorporated into the standard of care.[105–108] Indeed, numerous commercial high-throughput NGS assays are being developed, such as those available through Foundation Medicine. The current assays are

focused on detecting predefined actionable mutations, these companies are improving the existing technology to sequence formalin-fixed, paraffin-embedded tissue with a lower DNA requirement and at higher depth and will likely move towards whole-genome sequencing in the near future.

Non-invasive methods for sequencing DNA from alternative sources than the primary or metastasic tumour, such as circulating tumor cells, plasma DNA, exosomes and urine, are also being explored for diagnostic and predictive biomarker detection.

NGS for DNA and RNA have been used to sequence prostate cancer samples of different stages, for the study of mutations, amplifications, deletions and translocations and gene deregulation, respectively; this has increased our knowledge of the genomic landscape of prostate cancer and a movement toward developing a molecular new biomarkers as well as new therapeutic targets.[73,106]

Similar to genomic studies, research in cancer epigenetics has rapidly moved from candidate gene approaches to large-scale epigenomic approaches for characterisation of global epigenetic alterations in cancer. Applications of NGS technologies in epigenetics include transcriptome and small RNA sequencing (for example, miRNAs), readout platforms for ChIP (histone modifications, histone modifiers, histone variants, DNA methylation and chromatin remodellers) and whole bisulphite genomic sequencing (DNA methylation) experiments (Table 2). The use of NGS instead of custom-designed arrays to study the epigenetic landscape provides unbiased genome-wide information and has the potential to identify new biomarkers and better molecular targets for treatment in cancer.

Challenges for the use of epigenomic sequencing into the clinic. Epigenomic sequencing of large cohorts of cancer tissues, particularly for ChIP-seq and whole bisulphite sequencing, is quite behind compared with genomic sequencing. One of the main reasons is that the experiments are more time-consuming, there are limited bioinformatic tools and that our knowledge in the cancer epigenomic landscape needs a deeper understanding.[47] Data analysis and computer infrastructure are one of the major challenges to overcome; NGS technologies will bring an enormous amount of genomic and epigenomic data from patient sample that would need to be interpreted to build a comprehensive database of correlations between genomic and epigenomic alterations, signaling pathways, and disease.[47] This will require intensive discovery and research work and extensive computational analysis and integration. At the same time, clinicians will need to document patient clinical states, disease progression, and therapeutic response and nonresponse in searchable electronic medical records so that the information can be correlated with patient genetics and epigenetics. Nevertheless, genome-wide DNA methylation and miRNAs/ncRNAs are getting into the lead of identifying epigenomic biomarkers in cancer, including prostate cancer.[47,109,110]

Several international consortiums like the International Human Epigenome Consortium,[111] BLUEPRINT, ENCODE[112] and the International Cancer Genomic Consortium are underway to achieve this task to generate reference maps of human epigenomes relevant to health and diseases, including cancer, to provide a high number of reference epigenomes. The production of these reference epigenome maps would suppose a big step towards the discovery and validation of epigenomic biomarkers for diagnosis and prognosis in cancer.

CONCLUSIONS AND FUTURE PERSPECTIVES

Epigenetic alterations are a common feature of prostate cancer and have an important role in prostate carcinogenesis as well as in disease progression. The implementation of new technologies, such as NGS for global epigenomic analyses and integration with genomic and transcriptomic data, will exponentially expand our understanding of prostate tumorigenesis and will yield more clinically informative epigenetic biomarkers to aid in disease stratification. Therefore, we purport that in the future both genetic and epigenetic lesions will be used 'hand in hand' to support clinicians in patient diagnosis, prognosis and therapy response in prostate cancer.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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