Biomarkers in Cancer

REVIEW

Promoter Methylation in Prostate Cancer and its Application for the Early Detection of Prostate Cancer Using Serum and Urine Samples

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Abstract: Prostate cancer is the second most common cancer and the second leading cause of cancer death in men. However, prostate cancer can be effectively treated and cured, if it is diagnosed in its early stages when the tumor is still confined to the prostate. Combined with the digital rectal examination, the PSA test has been widely used to detect prostate cancer. But, the PSA screening method for early detection of prostate cancer is not reliable due to the high prevalence of false positive and false negative results. Epigenetic alterations including hypermethylation of gene promoters are believed to be the early events in neoplastic progression and thus these methylated genes can serve as biomarkers for the detection of cancer from clinical specimens. This review discusses DNA methylation of several gene promoters during prostate carcinogenesis and evaluates the usefulness of monitoring methylated DNA sequences, such as *GSTP1*, *RASSF1A*, *RARβ2* and galectin-3, for early detection of prostate cancer in tissue biopsies, serum and urine.

Keywords: prostate cancer, early detection, DNA methylation, GSTP1, gal3

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Introduction

Prostate cancer (PCa) is the second most common cancer in men after skin cancer and the second leading cause of cancer death in men after lung cancer. In the United States, there are over 300,000 newly diagnosed cases each year, and about 40,000 patients die of the disease.[1] Approximately 90% of patients with advanced prostate cancer develop osseous metastases, which are difficult to eradicate. Patients with osseous metastases have a mean survival time of nine months to one year.[1] PCa can be effectively treated and cured, however, if it is diagnosed in its early stages (i.e. in stage I and II), when the tumor is still confined to the prostate. Combined with the digital rectal examination, the prostate specific antigen (PSA) test has been widely used to detect PCa (Cancer Facts, National Cancer Institute; http://cis.nci.nih.gov/fact/5_29.htm). This test measures the serum levels of PSA, an enzyme that is produced by the prostate and released into the bloodstream, reaching concentrations no more than 3–4 ng/ml in healthy individuals. PSA levels above that value are considered as an indication of possible PCa. However, PSA is specific for prostate tissues, but not for PCa. Various non-cancerous conditions such as benign prostatic hyperplasia (BPH), prostatitis, prostatic ischemia or infarction can cause elevated levels of PSA (http://cis.nci.nih.gov/fact/5_29.htm). Further, serum PSA levels are not always a sensitive indicator for PCa, as these may be normal despite the presence of the disease.[2] Thus, the PSA screening method for early detection of PCa is not reliable due to the high prevalence of false positive and false negative results (sensitivity 90%; specificity 10%–31%).[3] Consequently, only 25 to 30 percent of men who have prostate biopsies based on elevated PSA levels are diagnosed with PCa.[4] Recently, assays based on the detection of the specific serum marker EPCA-2 (sensitivity 94%, specificity 92%)[5] and overexpression of telomerase (sensitivity 58%, specificity 100%), DD3 gene (sensitivity 67%, specificity 83%),[3] and prostate cancer gene 3 (PCA3) (sensitivity 58%, specificity 72%)[6] have been established and bear great promise for PCa diagnosis, and may reduce the number of unnecessary biopsies.

Epigenetic alterations, including hypermethylation of CpG islands in the gene promoters are believed to be early events in neoplastic progression.[7–12] However, recent findings in prostate carcinogenesis provide evidence that DNA hypomethylation changes occur subsequent to CpG island hypermethylation in later stages of carcinogenesis.[13] Hypermethylation of tumor suppressor gene promoters contributes to their silencing during the neoplastic process.[14] Thus, methylated gene promoters can serve as markers for the detection of cancer from clinical specimens such as tissue biopsies or body fluids.[15] Compared to tests that measure cancer-related proteins or RNAs, tests that measure gene alterations at the DNA level have several advantages for the early detection of cancer. DNA is stable in many of the conditions under which clinical specimens are collected and stored. Many DNA modifications can be reliably detected by PCR-based techniques,[16,17] meaning that very small amounts of DNA are needed for such tests. PCR amplification-based tests also allow detection of as few as one cancer cell (or genome copy) in a background of thousands of normal cells, thereby permitting detection of a cancer before it can be visualized by imaging or traditional pathology. Moreover, DNA alterations can be measured qualitatively, as well as quantitatively. Finally, assays based on the DNA alterations can be both diagnostic and prognostic. Therefore, methylated DNA sequences can form the basis of a sensitive and specific, robust and informative test for the detection of cancer.[17]

Alterations of DNA Methylation During Carcinogenesis: Hypomethylation in the Introns and Hypermethylation in the Promoter

DNA methylation refers to the covalent binding of a methyl group specifically to the carbon-5 position of cytosine residues of the dinucleotide CpG (Fig. 1). This is catalyzed by a family of enzymes, the DNA methyltransferases (DNMTs). Two types of DNA methylation alterations have been demonstrated in human cancers. The first refers to global hypomethylation in which the genomes of cancer cells show decreased methylation compared to normal cells.[18–20] The hypomethylation is primarily due to the loss of methylation in repetitive elements and other non-transcribed regions of the genome. This genome-wide hypomethylation potentially leads to loss of imprinting, chromosomal instability, cellular hyperproliferation, and activation of oncogenes[21] such as K-ras and PU.1.[22–25]

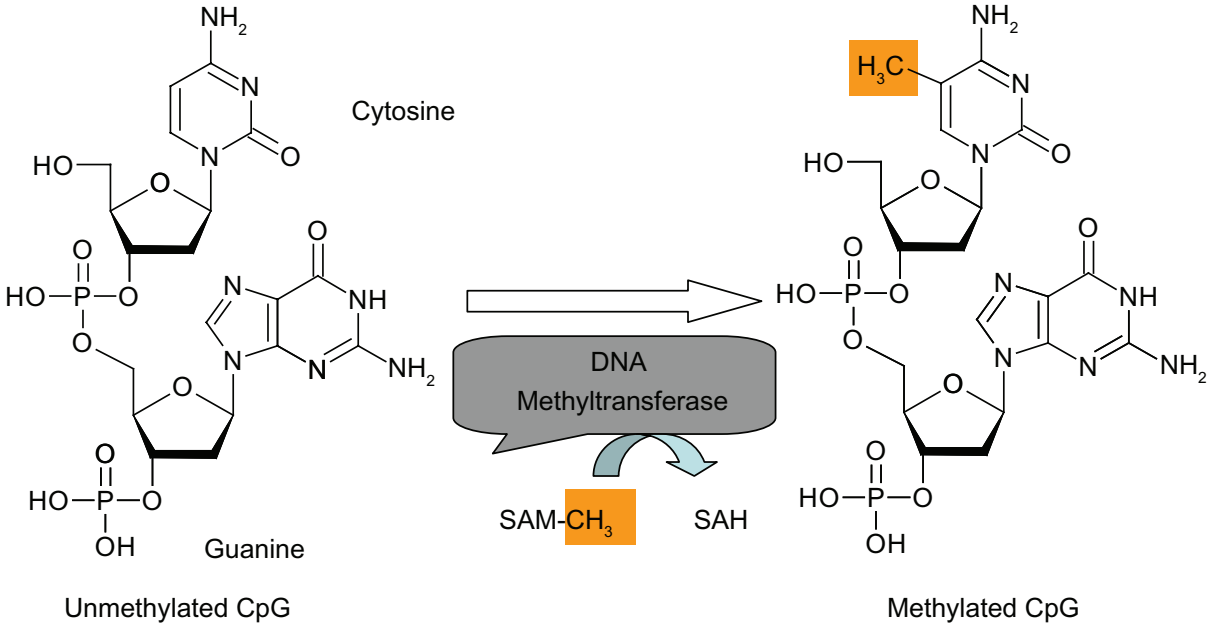

Figure 1. DNA methylation catalyzed by DNA methyltransferase. DNA methyltransferase transfers methyl group from S-adenosyl methionine (SAM-CH_3) to cytosine yielding S-adenosyl homocysteine (SAH) and 5-methylcytosine.

The second type of methylation alteration in cancer cells is the hypermethylation of CpG islands in the promoter regions of tumor suppressor and other regulatory genes that are normally unmethylated. The promoter regions of these genes may be inactivated by methylation, which silences their expression (Fig. 2). However, differential methylation is not a general mechanism for regulating gene expression, because most inactive promoters remained unmethylated.[26] It is thought that DNA methylation alters chromosome structure and defines regions for transcriptional regulation. Clusters of CpG sites are found dispersed around the genome and are referred to as CpG islands.[27] These islands are found in the promoter region of about 60% of genes, and in exons, introns, and repetitive elements of most genes. In normal cells, most CpG islands in the promoter regions are unmethylated whereas CpG islands in intronic regions and repetitive elements are heavily methylated, perhaps to help the cell identify regions for gene transcription.

Although the importance of CpG island methylation has been demonstrated in cancer, the mechanisms that lead to these changes in cancer are not yet understood. Of three members (DNMT1, DNMT3a, and DNMT3b) of the DNA methyltransferase family, DNMT1 is believed to be primarily involved in the maintenance of CpG methylation.[28,29] However, other studies suggest that DNMT3b, independently or in cooperation with DNMT1, also contributes to hypermethylation.[30–32] The suppression of transcription by DNA methylation may occur by either direct inhibition[33] or indirect inhibition[34] of transcription factor binding. For the latter, a family of proteins known as methyl binding domain (MBD) proteins is believed to specifically bind DNA containing methylated CpG sites.[34] At least three of the five known members of this family (MeCP2, MBD2 and MBD3) have been shown to be associated with large protein complexes containing histone deacetylase (HDAC1 and HDAC2) and chromatin-remodeling (Sin3a and mi-2) activities.[35,36] Histone deacetylase (HDAC1 and 2) and chromatin remodeling activities (Mi-2 and Sin3a) produce alterations in chromatin structure that make it refractory to transcriptional activation.[37] In addition to the large protein complexes, the MBD proteins may associate with several other complexes involved in transcriptional repression. Recently, MeCP2 was shown to interact with at least two other proteins, c-ski and N-CoR, known to be involved in transcriptional repression.[38] However, Ohm et al recently hypothesize that the stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable gene silencing during tumor initiation and progression.[39]

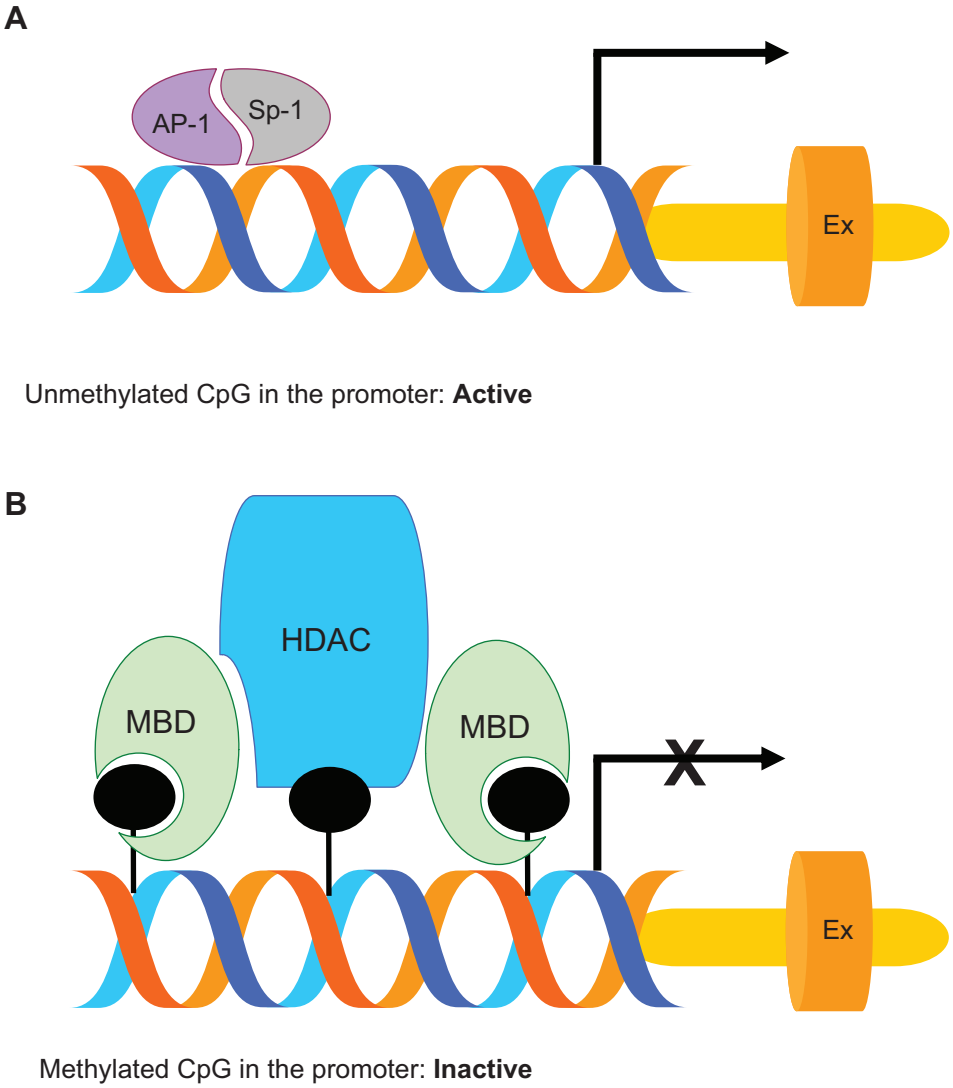

Figure 2. Simplified cartoon showing gene transcription by unmethylated promoter (A) and gene silencing by the methylated promoter (B). **A**) In normal prostate and pituitary tissues, tumor suppressor promoter is unmethylated and accessible to binding to the transcription factors such as AP-1 and Sp-1 for stimulation of gal3 transcription. **B**) In prostate cancer, promoters of several genes such as tumor suppressor, DNA repair gene, and gal3 are methylated and therefore bound by the methyl binding proteins (MBD) and histone deacetylase (HDAC). Thus the methylated promoter is not accessible to binding to the transcription factors and inactive.

As mentioned above, cancer cells exhibit two apparently opposing changes in the DNA methylation pattern: a decrease of DNA methylation in the intronic CpG islands and an increase of DNA methylation in the promoter CpG islands. Recent studies suggest that both changes may play important roles in the tumorigenic process. However, the increased methylation at the promoter CpG islands has been by far the most studied and has a much clearer role in carcinogenesis. Increased CpG island methylation can result in inactivation of many well-characterized tumor suppressor genes (e.g. *BRCA1*, breast cancer 1 gene) as well as inactivation of DNA repair genes, resulting in increased levels of genetic damage. The most striking example is the pi isoform of glutathione S-transferase (*GSTP1*), which is involved in detoxification of potentially DNA-damaging electrophiles.[40]

Hypermethylated Genes in Prostate Cancer

In prostate cancer, a large number of genes (e.g. DNA damage repair genes, tumor-suppressors, cell cycle control genes, cell adhesion molecules, and signal transduction genes) contribute to initiation and progression of the disease and expression of these genes is correlated with the pathological grade.[12,15,41–45] Interestingly, expression of some of these genes is regulated by DNA methylation at their promoters.

In general, promoter hypermethylation is associated with the loss of expression of these genes. A spectrum of methods is available for DNA methylation. These include cytosine deamination PCR, semi-quantitative and quantitative methylation-specific PCR (MSP), differential methylation hybridization (DMH), restriction landmark genomic scanning (RLGS), single-nucleotide primer extension (SNuPE), pyrosequencing, and methylation microarray for large-scale genome analysis.[15,46] However, MSP is a simple and sensitive method, and is the most commonly employed method for methylation analysis.[15] Table 1 shows some common genes that are hypermethylated in the prostate cancer and are tested for the development of early detection. These genes participate in DNA damage repair (*GSTP1*, *MGMT*), cell adhesion (*CD44, EDNRB, ECADHERIN, APC, LGALS3*), cell growth, invasion and metastasis (*TIMP2, TIMP3, LGALS3*), apoptosis (DAPK), cell cycle control (*CDKN2A, CDKN1A*), signal transduction (*RASSF1A*), and hormonal responses (*AR, ER, RARβ2*).[12,15,41–45]

The glutathione S transferases (GSTs) are a family of enzymes involved in protecting cells from DNA damage, and thence cancer initiation. GSTs carry out intracellular detoxification of xenobiotics and carcinogens by covalent bonding to glutathione via a thiol link.[47] In humans, six cytosolic isoforms of GST (alpha, mu, pi, sigma, theta, and omega) and one membrane isoform of GST have been described- of which the pi isoform (*GSTP1*) has been extensively studied.[48,49] In prostate cancer, *GSTP1* is observed to be silenced by promoter methylation.[17,39,50,51] *GSTP1* promoter methylation has been detected in cancerous as well as prostatic intraepithelial neoplasia (PIN) lesions, whereas it has been rarely detected in normal prostate or BPH tissues.[40,52,53] Hypermethylation of *GSTP1* was also found in a subset of proliferative inflammatory atrophy (PIA) lesions, which are believed to be tumors precursors.[53]

Another DNA repair gene O^6-Methylguanine-DNA-Methyltransferase (*MGMT*) was found to be hypermethylated (moderate to high levels) in many cancers including prostate cancer.[54,55] *MGMT* removes mutagenic and cytotoxic alkyl adducts from O^6-guanine in DNA.[56] However, hypermethylation of this gene promoter results in a loss of function in various cancers including prostate cancer.[55,57]

CD44, an integral membrane glycoprotein, plays a role in cell adhesion and cell-matrix interactions as a receptor for hyaluronic acid and osteopontin.[58] In prostate cancer, CD44, acts as a metastasis suppressor gene, and its down-regulation is associated with tumor progression and metastasis. Hypermethylation of CpG islands in the promoter region of CD44 results in decreased expression.[59,60] In a recent study, methylation of CD44 was evaluated among men who develop biochemical PCa recurrence after receiving radical prostatectomy.[61] The methylation profile of CD44 was found to be an independent predictor of biochemical recurrence (associated with 9-fold increased risk). This finding, if validated in larger studies, may identify patients with aggressive cancer.

The endothelin peptides consisting of three isotypes, ET-1, ET-2, and ET-3 have potent vasoconstructive properties and are differentially expressed in various cells and tissues.[62] Two receptors for endothelin peptides (ETA and ETB) have been identified in various cells and tissues. Belonging to a family of hyptohelical G-protein-coupled receptors, they are differentially expressed during prostate cancer progression and also differ in binding the enothelin isotypes.[63,64] ETA binds to two isotypes ET-1 and ET-2 only, whereas ETB binds to all three isotypes ET-1, ET-2, and ET-3.[63] In prostate cancer, expression of ETA is increased, whereas expression of ETB is reduced.[65] Moreover, the ETB gene (*EDNRB*) is frequently methylated in prostate cancer samples, but to a less so in benign samples.[66–68]

E-cadherin, a transmembrane glycoprotein and a member of the cadherin family of cell adhesion molecules, may function as a tumor suppressor gene in invasion and metastasis by mediating cell-cell adhesion via calcium-dependent interactions.[69] In prostate cancer, expression of E-cadherin is decreased during tumor progression and this decreased expression has been correlated with hypermethylation of the promoter in patients biopsies.[70,71] However, in metastatic prostate cancer cells in bone, E-cadherin expression is increased.[72] Interestingly, the promoter of E-cadherin gene (*CDH1*) is found unmethylated in the metastatic prostate cancer cells.[72]

The adenomatous polyposis coli (APC) is a multifunctional protein that acts as a tumor suppressor gene in familial adenomatous polyposis.[73] It plays a role in the Wnt signaling pathway, cell migration, cell adhesion,

Table 1. Summary of genes whose promoters are frequently methylated in prostate cancer.

Gene	Function	Methylation frequency	Stage of methylation	Marker for early detection?	Refs.
GSTP1	Intracellular detoxification (DNA damage repair)	70% to 100% (63/69)	All stages, also PIN; negative in BPH	May be suitable	40, 52, 53
MGMT	Removal of alkyl adducts from O6-guanine (DNA damage repair)	0% to 75% (8/32)	NA	May not be suitable as methylation is rare in prostate cancer	54, 55
CD44	Tumor suppressor: metastasis	33% to 78% (31/40)	Early to advanced, also in normal	May not be suitable	59, 60
EDNRB	Tumor suppressor	38% to 83% (40/48)	Frequent in prostate cancer, also in BPH	May not be suitable	66–68
CDH1	Tumor suppressor: invasion and metastasis	33% to 70% (19/35)	Mostly in advanced stages	May not be suitable	70, 71
APC	Tumor suppressor: invasion	27% to 38% (21/83)	All stages including PIN	May be suitable	75, 76
TIMP2	Tumor suppressor: invasion	60% (25/42)	Low levels in tumor, also in BPH and normal	May not be suitable	82–84
TIMP3	Tumor suppressor: invasion	6% to 97% (114/118)	Low levels in tumor, also in BPH	May not be suitable	82–84
DAPK	Apoptosis	1% to 36% (27/95)	Prostate cancer, also BPH	May not be suitable	75, 82, 86
RASSF1A	Tumor suppressor: cell growth	53% to 100% (74/95)	Frequent in early stages	May be suitable	75, 86, 96, 98–100
Cyclin D2	Cell cycle regulator	32% (32/101)	Frequent at higher stages, also in normal	May not be suitable	90
SFN	Cell cycle regulator	40% to 100% (41/41)	Prostate cancer, also in BPH	May not be suitable	93, 94
AR	Hormone regulation	0% to 28% (3/38)	Early and advanced stages, but low methylation frequency	May not be suitable	104–106
ERα	Hormone regulation	19% to 95% (35/38)	Early stages (high), metastatic (low), also in BPH	May not be suitable	106, 110–113
ERβ	Hormone regulation	20% to 100% (30/38)	Early stages (high), metastatic (low), also in BPH	May not be suitable	106, 110–113
SSBP2	Hematopoietic growth regulation	61% (54/88)	All stages, high methylation at advanced stages, negative in BPH	May be suitable	122
SLC18A2	Mono amine vesicular transporter	88% (15/17)	Negative in normal tissue, but positive in BPH (2 of 5 samples)	May not be suitable	124
MCAM	Melanoma adhesion	80% (70/88)	Higher methylation at advanced stages, also in BPH	May not be suitable	123
RARβ2	Tumor suppressor	30% to 97% (112/118)	primary and hormone-refractory tumors, also HGPIN, but not BPH	May be suitable	76, 117–120
gal3 (*LGALS3*)	Cell adhesion, tumor progression, anti-apoptotic	100% (18/18)	HGPIN, Stage I, II (heavy), Stage III, IV (light)	May be suitable	126, 127

Abbreviation: NA, not available.

and mitosis.[74] In prostate cancer development, *APC* hypermethylation has been observed in early cancer stages and in more than 30% of PIN samples.[75,76] The methylation frequency becomes higher as the disease progresses.[75,77] However, hypermethylation of the *APC* promoter was also observed in BPH tissues.[78]

Tissue inhibitors of metalloproteinases (TIMPs) are known to control the activity of matrix metalloproteinases (MMPs)[79] in several biological processes such as cell growth, apoptosis, invasion, metastasis and angiogenesis.[80,81] Four members of TIMPs have been identified and are known to be down-regulated in prostate cancer.[82,83] Down-regulation of TIMP proteins is associated with hypermethylation of the corresponding gene promoters.[84] In particular, low-level methylation of *TIMP2* and *TIMP3* promoters has been detected in prostate carcinoma as well as in BPH.[82–84]

Death-associated protein kinase (DAPK), a member of the pro-apoptotic calcium regulated serine/threonine kinases, is expressed in all tissues.[85] Its inactivation leads to the loss of this important apoptotic pathway. Although different mechanisms may affect *DAPK* inactivation in cancer, it has been shown that aberrant methylation is mainly responsible for silencing of the *DAPK* gene; inactivation of *DAPK* by promoter methylation has been observed in prostate cancer and BPH samples, but not in PIN samples.[75,82,86]

Cell cycle genes such as retinoblastoma protein (RB), cyclins, cyclin dependent kinases (CDKs), and CDK inhibitors (CDKIs) are very important in regulation of the cell cycle. In cancer, the efficacy of cell cycle checkpoints is often affected, especially control of the G1/S transition.[87] CDKIs are negative regulators of the cell cycle and considered to be tumor suppressor genes. CDKIs are categorized into two families, the INK4 family and the CIP/KIP (kinase inhibitor protein) family. The INK4 family is composed of four members CDKN2A or p16, CDKN2B or p15, CDKN2C or p18, and CDKN2D or p19.[88] The CIP/KIP family includes CDKN1A or p21, CDKN1B or p27, and CDKN1C or p57.[89] While the INK4 family specifically inhibits CDKs 4 and 6, the CIP/KIP family inhibits most CDKs.[88,89]

In prostate cancer, cell cycle checkpoint genes can be inactivated by a number of mechanisms such as deletion, point mutation, and hypermethylation. For example, cyclin D2 promoter methylation has been detected in prostate cancer and correlated with disease progression.[90] However, other cell cycle genes such as *p21* and *p27* are rarely methylated in prostate tumors.[91,92] Decreased expression of another negative cell cycle regulator 14-3-3sigma (SFN) due to promoter methylation has been detected in many cancers including prostate tumor and BPH.[93,94] Interestingly, in prostate cancer tissues, *p16* methylation has been frequently detected in exon 2 rather than in the promoter.[54]

RAS proteins are involved in extra-cellular signal transduction and regulate cell growth, survival and differentiation.[95] A new family of genes encoding RAS-binding proteins, RAS association domain family 1 gene (*RASSF1*), has been identified as a tumor suppressor in many carcinomas.[96] The *RASSF1* gene produces two predominant transcripts, *RASSF1A* and *RASSF1C*, that are regulated by distinct CpG promoter elements.[97] These transcripts are present in normal human tissues, but *RASSF1A* has been found to be inactivated in some prostate and other cancers.[96,98,99] Inactivation of *RASSF1A* at different stages of prostate cancer development is correlated with *RASSF1A* promoter methylation.[75,86,100]

Androgens such as testosterone and 5α-dihydrotestosterone are the main steroid hormones in the prostate and act through the androgen receptor (AR).[101] The expression of the AR gene and androgen dependence is consistent with the early stages of prostate cancer.[102,103] However, AR expression and androgen dependency is lost in the terminal stages of metastatic prostate cancer and the loss of AR expression appears to be regulated by DNA methylation in its promoter.[104–106] Moreover, methylation appears to be more prevalent in hormone-refractory tumors than in primary tumors.[105]

Estrogens are believed to play an important role in prostate carcinogenesis by acting through intracellular receptors, ER-α and ER-β.[107,108] These receptors are expressed in a cell and tissue specific manner, and involved in the regulation of the normal function of reproductive tissues.[109] However, several studies have reported the loss or down-regulation of these receptors during prostate cancer development[110,111] and the DNA methylation in their promoters has been associated with decreased or loss of expression of these two genes in prostate cancer.[106,112] Moreover, a high

frequency of methylation in the promoter region of the ER-β has been observed at the early stages of the disease, whereas methylation declined in metastatic tumors.[112] Promoter methylation of ER-α and ER-β in BPH has also been reported to a lesser extent than in prostate cancer tumors.[112.113]

RARβ2, an isoform of the β-subtype retinoic acid receptor, is expressed in most tissues and acts as a tumor suppressor gene.[114–116] In prostate cancer, expression of *RARβ2* is decreased or lost and this loss of expression is found associated with methylation in the promoter region.[76] Methylation of *RARβ2* in the promoter region has been frequently detected in PIN (low level), primary tumors, and hormone-refractory tumors (high level), but not in BPH and normal prostate.[117–120]

Single-stranded DNA-binding protein 2 (SSBP2), a novel regulator of hematopoietic growth and differentiation,[121] has recently been shown to be hypermethylated in prostate cancer.[122] In a quantitative MSP assay, the *SSBP2* promoter was hypermethylated in 61.4% of prostate cancer cases. In PIN tissues, *SSBP2* showed intermediate hypermethylation (30%), but no methylation in BPH.[122] Patients with tumors staging higher than pT3b (100%, 8 of 8) were found to be positive, indicating that *SSBP2* hypermethylation is associated with advanced tumor stage in prostate cancer.

The melanoma cell adhesion molecule (*MCAM*) gene promoter was recently found hypermethylated in prostate cancer (80%, 70/88) by quantitative MSP assay.[123] The *MCAM* promoter showed intermediate methylation in PIN (23%) and low methylation in BPH tissues (12.5). Like *SSBP2*, *MCAM* promoter methylation was directly correlated with tumor stage (pT3 + pT4) ($P = 0.001$) in primary prostate carcinoma.

The vesicular monoamine transporter 2 gene, *SLC18A2* was recently identified as a new target gene for CpG island hypermethylation in prostate adenocarcinoma.[124] SLC18A2 is an integral membrane protein of secretory vesicles, predominantly expressed in neurons and neuroendocrine cells, where it transports monoamines such as dopamine, serotonin, and histamine from the cytosol into vesicles for storage and/or exocytotic release.[125] *SLC18A2* hypermethylation was detected in 15 of 17 (88%) of prostate cancers examined. Methylation of *SLC18A2* was very minimal in 4 of 4 adjacent nonmalignant prostate glands and in 3 of 5 BPH samples, whereas the remaining 2 BPH samples had dense hypermethylation.[124]

The galectin-3 gene (*LGALS3*), a member of the galectin family, has recently been found to be hypermethylated in prostate adenocarcinoma.[126–128] Galectins, a family of β-galactoside-binding lectins, are multifunctional proteins involved in a variety of biological processes such as growth development, immune functions, apoptosis, and cancer metastasis.[129–131] Our studies and those of others indicate that galectins are transcriptionally regulated by DNA methylation.[126,132] Gal3 was found strongly expressed in normal, BPH, and high grade PIN (HGPIN) tissues, whereas expression of gal3 was found decreased in prostate adenocarcinoma.[127,133,134] By bisulfite sequencing of multiple prostate cancer specimens, the gal3 promoter of stage II tumors was seen to be heavily methylated throughout its entire length, but the gal3 promoter of stage III and IV tumors was lightly methylated. Whereas gal3 promoter in stage III showed few methylation sites, mostly between −199 to −252 nt, the gal3 promoter from stage IV tumor specimens was methylated between −112 to −227 nt. In stage I prostate cancer, however, both light and heavy methylation is evident in the gal3 promoter. In multiple normal prostate and BPH samples, the gal3 promoter was almost unmethylated. Overall, results indicated that the decreased expression of gal3 in tumor prostate is associated with the hypermethylation of its promoter.

Methylated Genes Suitable for Early Detection of Prostate Cancer

Although many genes are observed to be methylated in prostate cancer, a few genes have been investigated as targets for early detection (Table 2). Most have insufficient methylation frequency to provide the needed sensitivity, while other methylated genes are also present in the BPH, making them non-specific. The most suitable gene appears to be *GSTP1*, which is also the best studied in this regard. *GSTP1* promoter hypermethylation constitutes an ideal DNA-based biomarker for prostate cancer because it is present in up to 90% of prostatic cancer tissues and in 2/3 of high grade prostatic intraepithelial neoplasia (HGPIN) but not or rarely present in BPH tissue.[17,135–138] Other genes are

Table 2. Methylated genes used for early detection of prostate cancer from biopsy and biological fluid specimens.

Gene/Gene cohort	Specimen	Sensitivity %	Specificity %	Refs.
GSTP1	Biopsy	91 (10/11)	100	52
GSTP1	Biopsy	73 (11/15)	100	141
GSTP1	Biopsy	75 (46/61)	100	142
GSTP1, RARβ2, APC, TIG1	Biopsy	97 (59/61)	100	144
GSTP1	Biopsy washing	100 (10/10)	100	143
GSTP1	Ejaculate	44 (4/9)	NA	139
GSTP1	Ejaculate	50 (4/8)	100	140
GSTP1	Serum	72 (23/32)	100	140
GSTP1, PTGS2, Reprimo, TIG1	Serum	42–47	92	148
GSTP1, RASSF1, RARβ2	Serum	28 (24/83)	100	149
GSTP1	Urine	27 (6/22)	100	145
GSTP1	Urine post massage	36 (4/11)	100	140
GSTP1	Urine post massage	73 (29/40)	98	152
GSTP1	Urine post biopsy	39 (7/18)	NA	151
GSTP1, APC, EDNRB	Urine post biopsy	71 (12/17)	NA	153
GSTP1, INK4α, ARF, MGMT	Urine	87 (45/52)	100	154
GSTP1, INK4α, ARF, MGMT, RARβ2, TIMP3, CDH1, RASSF1A, APC	Urine	100 (52/52) (positive for at least one gene)	<100	154
GSTP1, RARβ2, APC, RASSF1A	Urine post massage	86	89	86
GSTP1, RASSF1A, ECDH1, APC, DAPK, MGMT, p14, p16, RARβ2, TIMP3	Urine post massage	93 (positive for at least one gene)	NA	86
GSTP1, RARβ2, APC	Urine	55	80	120
GSTP1, gal3	Biopsy	96 (26/27)	100	127
GSTP1, gal3	Serum	100 (4/4)	100	127
GSTP1, gal3	Urine	100 (22/22)	ND	Unpublished

Abbreviations: NA, not available; ND, not determined.

also used in combination with *GSTP1* in multiple-gene cohort assays. Using ejaculates, Suh et al first reported the presence of methylated *GSTP1* in 4 out of 9 patients with prostate cancer.[139] Later, Goessl et al found methylation in the *GSTP1* promoter in 72% (23/32) of sera, in 50% (4/8) of ejaculates, and in 36% (4/11) of urine samples from patients with prostate cancer, but none in any body fluid from 26 control patients with BPH.[140] A qMSP (quantitative methylation specific PCR) study by Jeronimo et al reported the highest sensitivity (90.9%) and specificity (100%).[52] By measuring the relative level of methylated *GSTP1* DNA to *MYOD1* (i.e. ratio of *GSTP1* to *myoD1* methylation) in sextant prostate biopsy samples from 21 patients with elevated PSA levels, the authors correctly predicted the histological diagnosis of prostate cancer in 90.9% (10/11) of the biopsies from patients with prostate cancer and confirmed negative prostate cancer in all 10 patients whose biopsy displayed no evidence of cancer. A subsequent analysis of *GSTP1* methylation further demonstrated the ability of qMSP to detect tiny

cancer foci in a large background of normal tissue.[141] Harden et al compared the results of blinded histological review of sextant biopsy samples from 72 excised prostates with those obtained from *GSTP1* qMSP.[142] Histology alone detected prostate carcinoma with 64% sensitivity and 100% specificity, whereas the combination of histology and *GSTP1* qMSP detected prostate carcinoma with 75% (46/61) sensitivity and 100% specificity. Another study using *GSTP1* promoter methylation on washings of needle biopsies from patients accurately detected prostate cancer (10 out of 10).[143] One advantage of this approach is that methylation analysis is performed on cells that accumulate in the needle during biopsy washings and the procedure does not impede routine histopathological assessment of the biopsy tissue.[143] However, the bottleneck of this approach is that several biopsies of an individual patient are needed for the test.[143] Other genes that are useful for early detection for prostate cancer include *RASSF1A*, *RARβ2*, *APC*, *AR* etc. (see Table 1), which are most often used in combination with *GSTP1*. For example, methylation analysis of a four-gene cohort (*GSTP1, RARβ2, APC*, and *TIG1*) resulted in the detection of 59 of 61 prostate cancer cases with 100% specificity, a 33% improvement over histology alone.[144]

The finding that the gal3 gene promoter is completely methylated in stage I and II PCa makes the gal3 gene (*LGALS3*) an ideal candidate for developing a methylation-specific PCR (MS-PCR) assay for early diagnosis of PCa.[126,127] Because stage I and II tumors are still confined to the prostate gland, identification of these stages is very important for effective intervention and cure. As the gal3 promoter is also methylated in stage III and IV, but only lightly and only between nt positions −112 to −252, we designed primers covering −9 nt to +64 nt to specifically detect stage I and II PCa (Fig. 3).[127] Of 34 tissues (5 normal, 2 BPH, 11 stage I, 7 stage II, 7 stage III, and 2 stage IV) tested, gal3 MS-PCR was positive with all stage I and II tumor samples (100% sensitive) on a semi-quantitative MS-PCR assay.[127] As expected, the gal3 MS-PCR was negative for normal, BPH, stage III (except one), and stage IV samples. In order to detect stage III and IV PCa samples along with the stage I and II, another assay based on *GSTP1* promoter methylation has been added with the gal3 MS-PCR. The combined MS-PCR assay (gal3 and *GSTP1*) detected 26 out of 27 prostate cancer tissues.[127]

Early Detection of Prostate Cancer in the Biological Fluids such as Serum and Urine

Several studies suggest that prostate cancer can be detected in serum and urine.[15,17,120,145] Identification of methylated DNA in urine would be a critical milestone in the development of a non-invasive diagnostic for early stages of prostate cancer. DNA passes into urine and blood through three main routes.[146] The first occurs when prostate cells are directly released into the urethra through prostatic ducts. DNA can also pass into urine by phagocytosis, in which macrophages engulf DNA from necrotic tumor tissue then the macrophages themselves appear in both urine and blood. Lastly, when cell proliferation is accelerated, cellular DNA content can overwhelm phagocytes and directly spill into circulation and urine.

The first study of body fluid-based detection involves the analysis of plasma samples of prostate cancer for the detection of *GSTP1* promoter hypermethylation. The *GSTP1* promoter was found hypermethylated in 72% (23/32) of PCa patients, but none of patients with BPH.[140,147] To determine the usefulness of multiple markers in serum samples, Ellinger and colleagues recently performed qMSP to measure hypermethylation of CpG islands in *GSTP1*, *TIG1*, *PTGS2*, and *Reprimo*.[147] These specific genes have been implicated in the pathogenesis of prostate cancer, and hypermethylation of these genes has been identified in prostate cancer tissue. All four genes displayed higher methylation frequencies in tissues of PCa patients (42.3%, 9.5%, 2.4%, and 1.2%, respectively) compared to BPH patients (7.7%, 0%, 0%, and 0%, respectively) and healthy controls (all 0%).[148] Comparing serum DNA of PCa and BPH patients, hypermethylation of either gene was highly specific (92%) but less sensitive (42%–47%).[148] Moreover, hypermethylation of *GSTP1* in serum identified 4 patients with incidental prostate cancer recurrence.[148] These studies indicate that the detection of *GSTP1* promoter methylation may serve as an additional tool to identify PCa in those patients with a high suspicion of disease despite negative biopsies. Very recently, Sunami et al[149] assayed blood from 40 healthy individuals and 83 patients with varying stages of PCa using MS-PCR

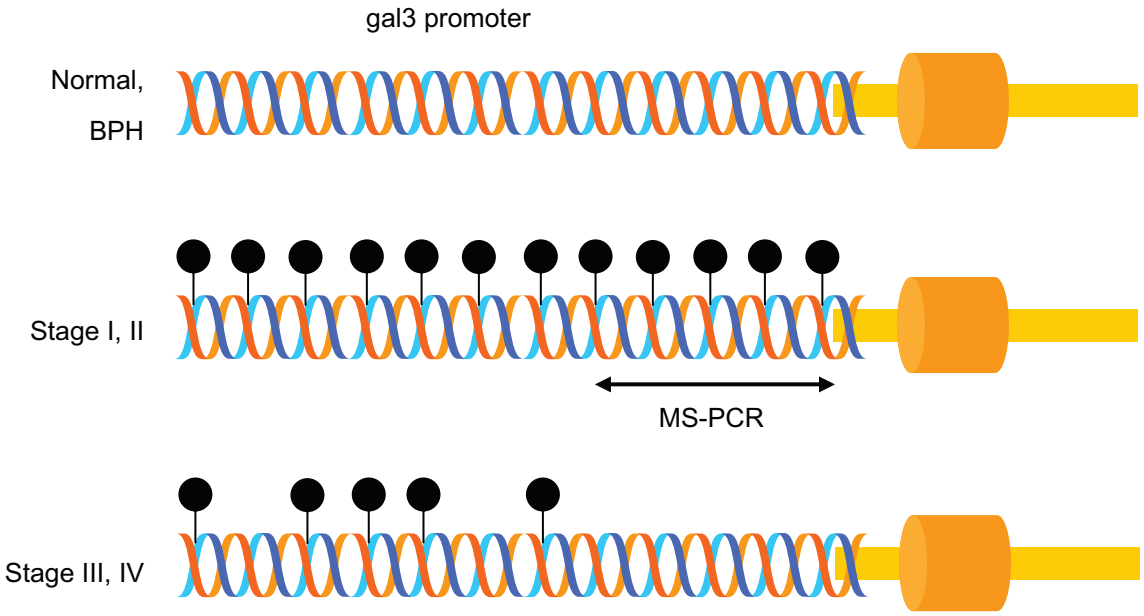

Figure 3. Schematic representation of MS-PCR. In normal and BPH prostate tissues, the gal3 promoter is unmethylated, whereas in stage I and II, it is methylated heavily. However, gal3 promoter is lightly methylated in stage III and IV. Stage-specific cytosine methylation of the gal3 promoter enabled the development of MS-PCR for the detection of stage I and II PCa.

of a 3-gene cohort (*GSTP1, RASSF1*, and *RARβ2*) and demonstrated detection of 28% cancer patients (24/83). However, a combination of the MS-PCR and PSA assays provided 89% sensitivity.

As described above, prostate cancers shed neoplastic cells or debris amenable to DNA analysis. Cairns et al[145] first analyzed voided urine samples from patients with prostate cancer and detected *GSTP1* hypermethylation in 27% of patients. The test was specific as patients with BPH were negative. Although *GSTP1* methylation was present in less than a third of PCa patient urine samples, the study showed for the first time that urine samples are amenable to DNA analysis. Later studies by Jeronimo et al[150] and Gonzalgo et al[151] showed *GSTP1* methylation in 23% and 39%, respectively, of urine samples from PCa patients. In another study, post-prostatic massage urine samples were assayed for the presence of *GSTP1* promoter hypermethylation resulting 75% sensitivity and 98% specificity for prostate cancer in contrast to biopsies which had 91% sensitivity and 88% specificity. This study indicates that detection of increased *GSTP1* methylation in urine samples may improve the specificity of PSA.

Goessl et al[152] demonstrated the utility of *GSTP1* methylation assays in urine sediments collected after prostate massage (sensitivity 73%; specificity 98%). Additional markers could potentially increase assay sensitivity. Rogers et al[153] evaluated the concordance between post-digital rectal examination (DRE) and post-prostate biopsy urine samples using conventional MS-PCR analysis of 3 gene promoters (*GSTP1*, *APC*, and *EDNRB*) in patients with suspected or confirmed prostate cancer. Prostate cancer was detected on prostate biopsy in 12 of 17 patients (71%). Promoter methylation of *GSTP1* (24%), *APC* (12%) and *EDNRB* (66%) was detected in post-DRE urine specimens. In post-biopsy urine specimens, methylation frequency of *GSTP1*, *APC*, and *EDNRB* was 18%, 18%, and 77%, respectively. The concordance between post-DRE and post-biopsy urine samples was 94% for *GSTP1* and *APC*, and 82% for *EDNRB*, suggesting that urine collected after DRE may be used for molecular analysis with results similar to those in post-biopsy urine samples.

An additional study in which multiple gene analyses were evaluated is that of Hoque et al,[154] who monitored a 4-gene cohort (*GSTP1*, *CDKN2A* (formerly *p16*), *PSCD2* (formerly *ARF*), and *MGMT*) that could theoretically detect 87% of prostate cancers at 100% specificity. However, *GSTP1* alone demonstrated a sensitivity of 48% at a specificity of 100%. In the same article, Hoque et al reported qMSP analysis of promoter methylation of 9-gene cohort (*p16*[*INK4a*],

p14[*ARF*], *MGMT*, *GSTP1*, *RARβ2*, *CDH1*, *TIMP3*, *RASSF1A*, and *APC*) in 52 urine sediment from PCa patients and 91 controls.[154] These 9 genes were chosen because their expression is frequently silenced by hypermethylation in prostate cancer.[154,155] Promoter hypermethylation was identified in at least 1 of the 9 genes in all 52 prostate cancer patients.[154]

Roupret et al[86] examined urine samples from 95 prostate cancer patients undergoing radical prostatectomy and from 38 control patients for aberrant methylation of 10 genes (*GSTP1*, *RASSF1A*, *ECDH1*, *APC*, *DAPK*, *MGMT*, *p14*, *p16*, *RARβ2*, and *TIMP3*). At least 1 gene was found hypermethylated in almost all cancer patients with a rare detection of *p14* (6.3%) and frequent detection of *GSTP1* (83.2%).[86] The 4-gene combination of *GSTP1*, *RASSF1A*, *RARβ2*, and *APC* best discriminated malignant from nonmalignant cases with a sensitivity of 86% and a specificity of 89%. However, these investigators used a 1-min prostate massage and bladder catheterization, which would hinder widespread adoption.

Vener et al[120] recently tested urine samples from 234 patients with PSA concentrations > or = 2.5 microg/L using 3 gene-cohort, *GSTP1, RARβ2,* and *APC*. In the first cohort of 121 patients, the authors demonstrated 55% sensitivity and 80% specificity; in the second cohort of 113 patients, they found a comparable sensitivity of 53% and specificity of 76%.

Our studies of gal3 MS-PCR in combination with *GSTP1* MS-PCR for a limited number of PCa patient serum[127] detected cancer in all sera (4/4), although more specimens should be tested for achieving a statistically significant dataset. Gal3 MS-PCR was also tested for a few urine samples from PCa patients and found positive in all specimens (22/22) (unpublished results).

Concluding Remarks

Several studies suggest that assays based on the detection of DNA methylation may provide better specificity and sensitivity than the PSA test. Furthermore, the methylated genes themselves may serve as promising biomarkers for early detection of prostate cancer. However, it should be emphasized that most published studies have been in small scale. Large scale studies will be necessary before any of theses assays can be considered clinically useful. In addition to clinical validation, assays for methylated genes must be robust, simple, sensitive, specific, and made available at affordable costs.

Regarding specificity of the assay, the most important consideration is to select a gene or genes whose promoter methylation is specific to prostate cancer. For example, *GSTP1* is methylated in 90% of prostate cancer, 30% of breast cancer, 25% of hepatic cancer, and less than 10% in bladder and renal cancers.[17] Given that prostate cancer is a disease of males and that hepatic cancer is relatively rare in the Western world, methylation analysis of *GSTP1* from male specimens would provide specificity to prostate cancer around 90%. Some other points are important to consider in order to improving specificity. Promoters of tumor suppressor genes are not typically methylated in normal cells, but methylated in tumor cells. Conversely, other genes, such as *myoD1* or *GDF15*, show low levels of methylation in normal cells and high levels of methylation in tumor cells.[156,157] Some genes such as maspin (*SERPINB5*) show tissue-specific methylation patterns in normal cells.[158] Moreover, some genes (e.g. *GSTP1*, *RARβ2*, *RASSF1A*) from normal cells of aging individuals are known to accumulate low-level promoters methylation.[159] Therefore, these genes should be investigated in age-matched negative controls to establish a quantitative cut-off point for the amount of methylation that would indicate the presence of cancer.

Regarding sensitivity, most studies of single genes or combinations of multiple genes did not reach a sensitivity of more than 75% and most importantly, these markers failed to detect early stages of prostate cancer.[120,144] Therefore, a reliable marker for early detection (stage I, II) of prostate cancer is yet to be identified. We have reported heavy promoter methylation of gal3 gene (LGALS3) in early stages of prostate cancer and have demonstrated the usefulness of *LGALS3* promoter methylation for early detection of prostate cancer (stage I and II) in a small number of specimens (tissue, serum, and urine)[127] (also unpublished results). Therefore, it is reasonable to propose that the quantitative measurement of gal3 promoter methylation in combination with that of *GSTP1* should provide both sensitivity and specificity approaching 100%.

The specificity of gal3 MS-PCR to prostate cancer is in question as gal3 is also known to be aberrantly expressed in many cancers. In most cancers such

as lung, liver, gastric, head and neck, thyroid, and bladder cancers, expression of gal3 is increased,[160–165] while expression is decreased in prostate, kidney, and pituitary cancers.[127,132,133,166] Although the loss of gal3 expression in pituitary tumor is partially due to promoter methylation, these methylation sites are distinct from those we observe in PCa by gal3 MS-PCR. Thus, by judicious primer design to target only the PCa-relevant regions the assay should be insensitive to pituitary cancer. Although it remains to be seen if the gal3 promoter is heavily methylated in kidney cancers, gal3 MS-PCR in a several urine specimens from kidney and bladder cancers yielded negative results (unpublished data) underscoring its specificity to prostate cancer. However, non-specificity of the assay can be substantially reduced by combining the assay with the conventional PSA test as explained below. The PSA test is complicated by a large number of false positive results (specificity 10%–31%) rather than the false negative results (sensitivity 90%). However, the PSA false positive data are due to non-cancerous conditions of the prostate such as BPH, prostatitis, prostatic ischemia or infarction, as the PSA is specific for prostate tissues. Thus combining the gal3/*GSTP1* cohort assay with the PSA test would increase the diagnostic power of the assay by reducing the gal3/*GSTP1* false positive results. With this combined test, four scenarios are possible. Scenario 1: High PSA and positive result with our gal3/*GSTP1* cohort assay = Confirmed prostate cancer; Scenario 2: Low PSA and negative result with gal3/*GSTP1* assay = No prostate cancer; Scenario 3: High PSA and negative result with gal3/*GSTP1* assay = No prostate cancer; and Scenario 4: Low PSA and positive result with gal3/*GSTP1* assay = May not be prostate cancer but may be positive with other cancer. The result of this last scenario may also represent indolent prostate cancer, depending on the gal3 selectivity for the indolent cancer yet to be established.

The selectivity of the assay for indolent prostate cancer bears some consideration. Indolent cancer (associated with low Gleason grade and a low PSA value) rarely becomes symptomatic during a patient's lifetime, and patients with indolent cancer are offered active surveillance rather than treatment.[167,168] However, a sensitive assay such as qMSP or "MethyLight" (detection limit 20 pg of DNA, equivalent to one cell or one genome copy) and early detection markers of prostate cancer identify a considerable proportion of cancers that are indolent.[167] There is a measurable risk, however, of over-treatment of patients with indolent disease, which should be avoided because of potential adverse psychological and somatic side effects.[168] Therefore, if the assay turns out to be positive with indolent cancer (samples with low PSA, Scenario 4 as described above), further tests (such as sarcosine marker)[169] should be performed to confirm the indolence behavior of the cancer prior to any treatment.

The prospect for improved detection of PCa with the gal3/*GSTP1* combined MS-PCR assay is promising. However, optimization of this combined assay and its validation in large scale studies are necessary before this combined assay can be considered clinically useful.

Conflict of Interest Statement

The author is one of the two inventors on a patent related to the application of galectin-3 promoter hypermethylation for early detection of prostate cancer.

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