Association of Tissue Promoter Methylation Levels of *APC, RASSF1A, CYP26A1* and *TBX15* with Prostate Cancer Progression

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract:

Aberrant promoter methylation is known to silence tumor-suppressor genes in prostate cancer. Using a quantitative real-time PCR assay (MethyLight), I determined promoter methylation levels of \textit{APC}, \textit{RASSF1A}, \textit{CYP26A1} and \textit{TBX15} in 219 radical prostatectomies diagnosed between 1998-2001, examined their correlation with clinicopathological follow-up data including Gleason Pattern (GP), Gleason Score (GS) and pathological stage, and explored their potential in predicting biochemical recurrence (BR) using univariate and multivariate analyses.

I demonstrated that methylation status of all four genes could accurately differentiate normal from cancerous tissues. Quantitative methylation levels of \textit{APC} and \textit{TBX15} correlated strongly with GP, GS, and pathological stage. Both \textit{APC} and \textit{TBX15} methylation levels could significantly predict BR in univariate analysis (p-value=0.028 and 0.003, respectively). The methylation profiles of \textit{APC} and \textit{TBX15} combined could discriminate patients into high, intermediate, and low risk groups of BR (p-value=0.005).

My project demonstrated that quantitative increase in promoter methylation levels of \textit{APC} and \textit{TBX15} were associated with PCa progression.
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**Abbreviations:**

- **APC**  *Adenomatous polyposis coli*
- **AUC** Area under the Curve
- **BR** Biochemical Recurrence
- **BPH** Benign Prostatic Hyperplasia
- **CGIs** CpG islands
- **Cobra** Combined Bisulfite Restriction Analysis
- **CYP26A1** Cytochrome P450, family 26, subfamily A, polypeptide 1
- **DMH** Differential methylation hybridization
- **DNMT** DNA Methyltransferase
- **DRE** Digital Rectal Examination
- **ERSPC** European Randomized Study of Screening for Prostate Cancer
- **GS** Gleason Score
- **GP** Gleason Pattern
- **HDI** Histone Deacetylase Inhibitor
- **HM** High Methylation
- **LM** Low Methylation
- **LNM** Lymph Node Metastasis
- **MGMT** O6-Methylguanine-DNA-Methyltransferase
- **PCa** Prostate Cancer
<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>PLCO</td>
<td>Prostate, Lung, Colon and Ovary cancer screening trial</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3 Kinase</td>
</tr>
<tr>
<td>PMR</td>
<td>Percentage of Methylated Reference</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>Ras association domain family member 1, transcript A</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic Acid</td>
</tr>
<tr>
<td>RARE</td>
<td>Retinoic Acid Response Element</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic Acid Receptor</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver Operating Characteristics curves</td>
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<tr>
<td>RXR</td>
<td>Retinoic X Receptor</td>
</tr>
<tr>
<td>TBX15</td>
<td>T-box 15</td>
</tr>
<tr>
<td>TRUS</td>
<td>Transrectal Ultrasound</td>
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Chapter 1 Introduction
1.1 Prostate Cancer

1.1.1 Incidence, Survival and Risk Factors

Prostate cancer (PCa) is the most common cancer diagnosed in Canadian men, with an estimated 24,600 new cases and 4,300 deaths expected in 2010 (Canadian Cancer Statistics 2010). The major risk factors for the development of PCa are advanced age, family history and genetic disposition [1], ethnicity (African ancestry), and environmental factors such as diet rich in well-done meats [2]. High-grade prostatic intraepithelial neoplasia (PIN) is believed to be the most likely precursor of prostatic adenocarcinoma [3]. PCa is characterized by the long latency period between the appearance of PIN and the manifestation of clinically detectable cancer. Post-mortem studies show that while a very high proportion of elderly men have histological evidence of the disease, a much smaller proportion develops clinically apparent cancer[4]. The annual incidence is likely to increase in the next decade due to an aging population, lower threshold for prostate biopsy, and increased uptake in PSA screening.

1.2 Prostate Cancer Screening and Diagnosis

Prostate cancer can be diagnosed by digital rectal examination (DRE) that checks for growth in or enlargement of the prostate gland in conjunction with serum prostate-specific antigen (PSA) screening. Abnormal DRE results and/or elevated serum PSA level is followed by transrectal ultrasound (TRUS), with histological confirmation by needle biopsy. The combination use of these tests helps clinicians to identify tumors that are confined within the prostate, and are thus potentially treatable. A general outline of the currently accepted protocol for early detection of PCa is presented in Figure 1.1.
Figure 1.1 Overview of early screening of prostate cancer.

 Candidates for early detection testing: Baseline PSA age 40 years with anticipated lifespan of 10 or more years

 What tests should be offered: Prostate specific antigen AND Digital rectal examination

 1. DRE abnormal/PSA low for age (consider possible causes: prostate cancer, BPH, infection, trauma, etc)
 2. PSA high for age or
 3. DRE abnormal and PSA high

 Both tests are low / not suspicious
 Return regularly for PSA and DRE

 Counsel patient regarding both risks and benefits of biopsy

 Biopsy done, extended, local anaesthesia

 Biopsy Positive

 Biopsy Not Done

 Management discussion and risk assessment

 Active surveillance or Treatment
1.2.1 Serum Testing for Prostate-Specific Antigen

In recent years, Prostate Specific Antigen (PSA) testing has led to a sharp increase in PCa incidence, with most patients presenting early with a clinically localized (low risk) disease. In the early 1990s, evidence revealed the potential effectiveness of serum PSA measurement as a useful adjunct to rectal examination and ultrasonography in detecting PCa [5]. The incidence to mortality ratio worldwide was 3:1[6] but this was shown to be greatly increased with the introduction of PSA testing to 7:1 in Canada (Canadian Cancer Statistics 2010).

PSA is a glycoprotein secreted exclusively by prostatic epithelial cells. It appears in the circulation as a result of epithelial damage or dysfunction in the prostate [7]. The serum PSA levels are normally very low and can be elevated in a variety of noncancerous prostate diseases and conditions, including prostate infections, inflammation, trauma, and benign prostatic hyperplasia (BPH) [8]. An elevated serum PSA level is not a PCa-specific event, and this results in a lack of specificity in PSA testing and consequently a high negative biopsy rate [9].

PSA continues to be the most important test for PCa diagnosis in Canada in spite of an increasing understanding of its associated disadvantages. Recently, two randomized studies were undertaken to examine whether PSA-based screening effectively reduces PCa mortality rates but yielded contradicting results. The European Randomized Study of Screening for Prostate Cancer (ERSPC) was initiated in the early 1990s, and included 182,160 men aged 50–74, with a median follow-up of 9 years through registries in seven European countries [10]. Subjects in the PSA-based screening group experienced a 20% significant reduction of PCa death compared to the control group [10], and this study provided evidence for benefits
associated with early detection testing. On the other hand, the prostate, lung, colon and ovary cancer screening trial (PLCO) found no significant difference in PCa mortality rate between the screening group and the control group [11]. The PLCO study was considered flawed due to the enrollment of a large proportion of men (>40%) who had undergone prostate-cancer screening in the previous 3 years of the study. Moreover, more than 50% of the control subjects underwent screening during the study [11].

Another downside of PSA screening is that there remains a considerable chance of patients being overdiagnosed and overtreated with consequent health care cost [6, 10, 12]. A significant fraction of the screen-detected PCa tumors might never give rise to clinical symptoms or result in patient death [13-15]. It has been reported that the performance of a PSA test can be improved by adding other risk factors such as prostate volume and outcome of DRE [16], or by examining the rate of change in PSA values over time (PSA velocity) [17, 18].

It is very important to calculate the sensitivity, specificity, positive predictive value and negative predictive value in order to determine how useful a diagnostic test, such as PSA testing, is to detect the presence of PCa tumors. Sensitivity measures the proportion of people who are correctly identified as harboring PCa tumors; specificity measures the proportion of healthy people (people without PCa) which are correctly identified; the positive predictive value measures the proportion of patients with positive test results who are correctly diagnosed; and finally, the negative predictive value measures the proportion of patients with negative test results who are correctly diagnosed [19]. A hypothetical example of the PSA screening test used to look for PCa cancer is illustrated in Table 1.1. A theoretical, optimal
prediction can achieve 100% sensitivity, 100% specificity and 100% positive and negative prediction values.

**Table 1.1** The relationship between sensitivity, specificity, the positive predicative value and the negative predicative value

<table>
<thead>
<tr>
<th>PSA Test Outcome</th>
<th>Patient Conditions</th>
<th>Positive (with PCa tumor)</th>
<th>Negative (without PCa tumor)</th>
<th>Positive predicative value = $A/(A+B)$</th>
<th>Negative predicative value = $D/(C+D)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>True Positive = A</td>
<td>False Positive = B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>False Negative = C</td>
<td>True Negative = D</td>
<td></td>
<td></td>
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</tr>
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</table>

Sensitivity = $A/(A+C)$
Specificity = $D/(B+D)$

1.2.2 Prostate Cancer Antigen 3 Test

Prostate Cancer Antigen 3 (PCA3) is an alternative screening tool that has been developed to improve specificity of early PCa diagnosis and help to determine candidate patients for biopsy. PCA3 is a prostate-specific non-coding RNA that was found to be highly overexpressed in 95% of prostatic tumors compared to normal prostate tissues, suggesting its diagnostic potential for PCa [9, 20]. PCA3 expression is reported to be prostate-specific since no expression of PCA3 could be detected in other normal human tissues, tumors, or various cancer cell lines except LNCap and 22RV1 [9, 20].

PCA3 expression level has been demonstrated by several studies to have an improved sensitivity and specificity over the PSA test for prostate cancer detection [21, 22], and a higher PCA3 score is indicative of a higher probability of a positive biopsy result [23].
(Figure 1.2). In addition, PCA3 score has been shown to correlate positively with PCa tumor size, clinical significance of tumors, and extracapsular extension [24, 25]. A transcription-mediated amplification (TMA) platform has been developed for fast and sensitive detection of PCA3 mRNA, enabling the potential diagnostic utility of PCA3 in biological fluids [21].

**Figure 1.2** Sensitivity and specificity of PCA-3 testing for prostate cancer detection.

![ROC analysis of PCA-3 testing](image)

**a) ROC analysis of PCA-3 testing**

**b) Comparison of the diagnostic efficiency of PCA-3 test and serum PSA level**

ROC refers to receiver operating characteristics curves


Even though PCA-3 is shown to be superior to PSA testing for predicting biopsy outcome, inconsistent cutoff values for PCA-3 score were employed by different study groups [22, 25-27]. The sensitivity and specificity were quite heterogeneous among various studies, ranging from 46.9% to 82.3% and from 56.3% to 89% respectively [22, 25-27]. The sensitivity of the PCA-3 test was often less than that of PSA testing, and studies suggested contradictory results regarding the use of PCA-3 to predict PCa prognosis. Therefore, it is imperative to
discover novel biomarkers that are capable of accurately identifying patients harboring PCa tumors.

1.2.3 Digital Rectal Examination (DRE)

DRE involves the insertion of a finger into the rectum to palpate the prostate gland for induration or abnormal masses. It remains an important early and relatively non-invasive investigative test that can be easily performed in a physician’s office. The false positive rates for DRE are relatively high (~40-50%) because lesions detected by DRE may be caused by a variety of conditions such as BPH, prostatic atrophy, etc. [28, 29] DRE is now unacceptable as a sole method for detecting prostate cancer due to its suboptimal sensitivity and specificity for PCa detection.

1.2.4 Transrectal Ultrasound Imaging and Needle Biopsy

Transrectal ultrasound imaging is used to confirm the diagnosis of PCa, estimate the size of the PCa, and guide needle biopsies. The estimation of prostatic volume may assist in evaluation of benign and malignant disease and can be used in the determination of appropriate therapeutic intervention[30]. Needle biopsy assists in the evaluation of benign and malignant disease and can be used in the determination of appropriate therapeutic intervention[4]. Normally, multiple biopsies are obtained from different locations within the prostate. For many years, the standard procedure of prostate biopsy involved removing 6 cores from the paramedian prostate regions [31]. However, several studies have argued that the traditional protocol misses 10%-30% of cancers and suggested the application of extended prostate biopsy scheme involving more tissue cores and varying anatomical regions [32, 33]. The combination of cores and areas is under constant modification to achieve
optimal cancer detection rate and minimize biopsy related complications. The new extended protocols increase the number of biopsy cores (from 12 up to 32 cores) and add laterally directed cores to the base and medially to the apex in addition to cores from the central gland to achieve significantly increased cancer detection rate [34, 35]. The tissue removed at biopsy is preserved and will be examined under a microscope to determine the presence of PCa.

1.2.5 *The Gleason Grading System*

Tumor differentiation is graded using a Gleason Score (GS), which is consistent and highly correlated with patient outcome. Gleason Patterns (GP) are assigned the most common histological tumor cell patterns exhibited by the cancers, and GS is the sum of two most prevalent GPs [9]. GP ranges from 1 to 5, with 5 being the most poorly differentiated, and GS therefore ranges from 2 to 10 [36] (Figure 1.3). Prostate tumors with GS\(\leq 6\), usually composed of GP3, are defined as low-grade cancers and tend to be slow-growing and localized. GS7 tumors, constituted by a mixture of GP3 and GP4, are considered intermediate, while tumors with GS\(\geq 8\) represent high-grade cancers with fast-growing and aggressive phenotype, which are likely to metastasize [36].
Figure 1.3 Gleason Patterns from 1 to 5 based on glandular architecture of the prostate


### 1.3 Treatment Options for Prostate Cancer

#### 1.3.1 Treatment Options for Localized Prostate Cancer

Three major treatment options are available for clinically confined PCas: active surveillance, radical prostatectomy and radiotherapy. Active surveillance, also referred to “watchful waiting” is a conservative treatment that is usually reserved for non-symptomatic patients with PCa, particularly for older men with relatively short life expectancy [37]. Patients undergoing watchful waiting are usually reviewed regularly with PSA screening and DRE to assess disease progression and discuss treatment options. Radical prostatectomy has become safer and widely performed due to improvements in surgical techniques resulting in nerve-sparing, reduced blood loss and fewer post-operative complications. It is mainly reserved for locally confined PCas, but is not an optimal choice for PCa patients with tumors that have
metastasized to other parts of their body. The overall survival rate is reported to be higher for men treated with radical prostatectomy than for men managed conservatively[38]. Radiation therapy including external beam radiation, radioactive seed implants, and brachytherapy have also been used widely to PCa. It appears that survival and recurrence rates following radiotherapy are strongly related to grade and stage of the disease. The probability of curative treatment following radiotherapy is highest for organ confined, small volume, and well-differentiated tumors. Combined radiotherapy and hormone therapy are being designed for locally advanced prostate adenocarcinomas [39].

1.3.2 Hormone Therapy for Advanced or Metastatic Prostate Cancer

Androgen deprivation therapy has been the major treatment of men with advanced stage PCa and has been associated with prolonged life span [40]. However, patients with metastatic disease ultimately become unresponsive to therapy and castrate-resistant PCas cannot be adequately addressed by currently available therapies [41]. Several possible mechanisms have been proposed for the emergence of hormone refractory PCa: 1) molecular alterations occur during the PCa tumorigenesis and give rise to androgen-independent cells which outcompete others during androgen ablation [42]; 2) there are preexisting androgen-independent stem cells that undergo clonal selection [42, 43]. Nevertheless, late-stage and generally lethal hormone refractory PCa poses a major challenge to clinicians. Alternatives to classical androgen deprivation therapy, such as intermittent androgen suppression, administration of novel androgen synthesis inhibitors, and estrogen therapy, are proposed to better suppress androgen levels and androgen-receptor-mediated effects while improving toxicity profiles [44]. Therapeutic interventions are also being developed to target aberrant
activated pathways in castration-resistant PCas and at the surrounding microenvironment of the tumor [45].

1.3.3 Clinical Predictive Models for Prostate Cancer

Accurate estimation of PCa clinical significance, potential treatment-induced complications, and long-term morbidity are critical for optimal patient care. There are a variety of predictive models including 1) probability tables, 2) risk groupings, 3) classification and regression tree (CART) analyses, 4) nomograms, and 5) artificial neural networks that greatly facilitate clinicians in decision-making [46, 47]. Depending on the various clinical scenarios that need to be addressed, ranging from predicting biopsy outcomes in men with elevated serum PSA [48, 49] to death from hormone-refractory cancers[50], one specific model is preferred over others to give higher predictive accuracy and better performance characteristics. The general model selection criteria include a) the predictive accuracy of the model, b) the level of complexity (user-friendliness), c) generalizability across populations and in various clinical scenarios, d) calibration, which allows evaluating the underestimation and overestimation of the predictions relative to the observed rates, and finally e) advantages and disadvantages relative to established alternatives[51]. The existing preoperative nomograms, which are commonly composed of three clinicopathological variables including Gleason score, disease stage and PSA level, are beneficial but are suboptimal for selection of effective treatment decisions due to clinical heterogeneity of prostate cancer[52]. Better understanding of the biology of prostate cancer progression is needed to predict more accurately which patients will likely to respond to specific targeted therapeutic interventions.
1.3.4 Predictive Molecular Markers of Response to Prostate Cancer Therapy

Establishing predictive markers would help to anticipate patient response to therapeutic interventions and individualize therapy. Understanding the molecular characteristics underlying an individual’s tumor is fundamental in making informed treatment decisions.

PSA level has long been used as a predictor of treatment failure after cryoablation or high-intensity focused ultrasound [53, 54]. In addition, PSA doubling time has been suggested as a good indicator to determine treatment options for clinically localized tumors: watchful waiting versus radical prostatectomy.

Androgen-deprivation therapy continues to be the most important treatment for advanced PCa. Although patients initially respond favorably to it, PCa tumors eventually progress to a castration-resistant state despite maintenance of androgen-ablation and become fatal. Therefore, it is important to understand how an individual’s genome impacts response to treatment. Three favorable single-nucleotide polymorphisms within three genes, CYP19A1, HSD3B1 and HSD17B4, have been reported to be associated with significantly longer duration of response to hormone therapy [55]. The above results shed light on the pathways that govern response to androgen ablation therapy and could help predict therapeutic efficacy. In an attempt to individualize therapy for castration-resistant prostate cancer (CRPC), a transcription-based AR activity signature was developed to detect AR activity within individual prostate cancer specimens and demonstrated that CRPC are heterogeneous in their level of AR activity [56]. This AR signature will help stratify patients into two groups: patients with high AR activity who would benefit from further AR-targeted therapy and patients with low AR activity who should receive Src-targeted therapy [57].
Finally, predictive markers that could potentially mediate radiotherapy and chemotherapy resistance are defined in PCa. In a phase 2 neo-adjuvant docetaxel trial for men with high-risk, localized PCa, elevated expression of genes that are involved in metabolizing bioactive androgens was observed in tumors after docetaxel therapy compared with untreated control tumors [58]. This finding provided support of androgen signaling being involved in PCa patient response to docetaxel. Many past studies have also reported that the overexpression of bcl-2 and mutated p53 might mediate resistance mechanisms to the apoptotic effects of radiation [59-61]. In the future, routine patient care could include using the molecular signature of a PCa patient's tumor to guide treatment.

1.4 Diagnostic Dilemma

Among all preoperative covariates, GS is the most predictive of biological aggressivity [36, 62]. Despite its astonishing performance, there are three problems that have limited the effective application of Gleason Grading system to risk stratification. First of all, histopathologically similar PCas are biologically diverse: some tumors remain indolent for many years while others progress rapidly to invade other parts of the body. In particular, patients with GS 7 tumors, composed of predominant GP 4 and lesser contribution of GP 3 (4+3), have in general more aggressive disease and experience high rates of systemic recurrence and cancer specific death than those with a predominant pattern 3 (3+4) [63]. Second, any histological grading system possesses an inherent degree of subjectivity and consequently, both intra- and inter-observer variability to accurately assign GS exist [64]. Finally, as a consequence of sampling bias, GS obtained on a biopsy often underestimates the GS in the corresponding prostatectomy specimen, resulting in inappropriate treatment choices [65-67]. This poses a major diagnostic dilemma to clinicians because based on GS
alone, fast progressing tumors cannot be accurately identified from clinically insignificant tumors, leading to the possibility of some patients receiving inappropriately aggressive local therapy while others are prevented from receiving adequate therapeutic management. The notion underlines the urgent need for advanced novel biomarkers capable of objectively separating indolent from aggressive PCas.

1.5 Somatic Molecular Alterations in Prostate Cancer

A large number of somatic genome alterations, both genetic and epigenetic, have been characterized to contribute to the tumorigenesis of PCa. Genetic alterations include point mutations, chromosomal deletions, gene amplifications, and translocations. Several well characterized genetic changes are detailed below.

1.5.1 TEMPRSS2-ERG Gene Fusion

Fusion of the 5’ untranslated region of an androgen-regulated prostate-specific serine protease, TMPRSS2, with members of the ETS transcription family, is one of the most frequently reported chromosomal rearrangements in PCa [46, 47, 68, 69] (Figure 1.4). ETS is a family of transcriptional activators and inhibitors with four members ERG, ETV1, ETV4, and ETB5[70]. TMPRSS2 is under the regulation of androgens in prostate; therefore gene rearrangement leads to overexpression of ETS oncogenes. The TEMPRSS2:ERG is the most common fusion product, with over 20 variants described to date[71]. PCa is a heterogeneous cancer and tends to be multifocal. Different foci from the same tumor might harbor rearrangements involving different genes, or no gene fusion products at all [72-74].
Past studies have attempted to assess the prognostic significance associated with the fusion construct \textit{TEMPRSS2: ERG} by correlating with clinicopathological features. The presence of the fusion protein was found to correlate with tumor stage and grade but could not serve as an adverse prognostic marker [72, 75, 76]. Later studies reported contradictory finding that translocation resulting in \textit{TEMPRSS2: ERG} is associated with PCa patient mortality [77, 78]. The \textit{TEMPRSS2: ERG} is specific to prostate cancer and may have important implications in understanding PCa tumorigenesis and developing potential diagnostic therapeutics.

**Figure 1.4** Formation and consequence of TMPRSS2-ERG gene fusions


**1.5.2 Telomere Shortening**

Chromosomal instability has been implicated in the pathogenesis of malignant transformation in human cancers. Telomeres are repeat DNA sequences that stabilize and protect the ends of
chromosomes. Telomeres become markedly shortened in cancer development, leading to increased incidences of chromosome fusions, subsequent breakage, and rearrangement [79, 80]. It has been shown in the human prostate that the telomere lengths of HGPIN lesions and PCa tumors were significantly shorter than those of adjacent normal cells [81, 82]. Therefore, telomerase shortening may be a consistent feature of PCa tumorigenesis and may be associated with promoting chromosomal instability leading to cancer development.

1.5.3 Loss of Heterozygosity of Tumor Suppressor Genes

Chromosomal abnormalities of deletions at 8p, 10q, 13q, and 16q occur frequently in PCa [81, 83]. Regions of commonly allelic loss have been reported to contain tumor suppressor genes. \textit{NKX3.1}, which localizes on the frequent deleted region of chromosome 8p, encodes a homeobox protein that is involved in the normal differentiation of the prostatic epithelium. Its loss of expression in PIN lesions and in prostate tumor cells might serve as an initiating event in prostate carcinogenesis [84, 85]. Another example is the \textit{PTEN} gene on 10q23, which is located to another frequently deleted region in PCAs. It encodes a lipid phosphatase that negatively regulates the phosphoinositide-3 kinase (PI3K)/Akt signalling and prevents uncontrolled cell growth [86, 87]. Many studies have reported cooperative activity between the androgen receptor and the PI3K/Akt signalling pathways in regulating PCa tumor development and progression [88, 89]. It seems that AR transcriptional activity and expression are regulated by Akt [88]. Loss of \textit{PTEN} is significantly associated with high GS and advanced stage[82]. Compound mutants of \textit{NKX3.1} and \textit{PTEN} can develop metastases and androgen independence with increasing age [90, 91]. Genetic inactivation of the classic tumor suppressor genes, such as retinoblastoma (\textit{Rb}) and \textit{TP53}, occur rarely in early tumors, but are present in approximately 20–50% of advanced-stage PCAs[92].
1.5.4 Amplification of Androgen Receptor Signalling

Prostate cancer cells require the presence of androgen or androgen derivatives to survive. Under normal conditions, in the absence of hormone, androgen receptor (AR) is sequestered in the cytoplasm and prevented from entry into the nucleus and activation of gene transcription [93]. Upon binding to androgen, AR is dissociated from the cytoplasmic complex, resulting in nuclear translocation and signal amplification [93]. The action of AR is under tight regulation of co-activators and co-repressors [94]. Several functional mechanisms have been proposed to explain aberrant AR signalling pathway activation in castration-resistant PCa tumors that are depleted of androgen[93]: 1) AR amplification and/or overexpression [95, 96]; 2) AR mutations that affect ligand-receptor binding or protein-receptor interactions, leading to gain of function [97]; 3) synthesis of exogenous, intra-tumor androgens [98]; 4) activation of AR in a ligand-independent manner via cross-talk with growth factor receptors. End-stage, androgen-independent tumors still rely on an active AR signalling pathway for growth and survival, and that makes AR an optimal target for therapeutic interventions.

1.6 Epigenetic Alterations in Prostate Cancer

Epigenetic mechanisms permit the stable inheritance of cellular properties without changes in DNA sequence. Epigenetic mechanisms include methylation of CpG dinucleotides, histone modifications, such as methylation or acetylation of histone tails, RNA-mediated gene regulation, and chromatin remodeling [99]. DNA methylation refers to the addition of a methyl group to the 5’- carbon of cytosine in the context of CpG islands. CpG islands (CGIs) are short stretches of DNA (~1,000 bp) in which CG content is elevated and have been
shown to frequently colocalize with the transcription start sites of 60%-70% human genes[100, 101]. During PCa development, locus-specific aberrant promoter hypermethylation is associated with early stage cancer development, while global DNA hypomethylation, especially loss of methylcytosine at retrotransposon repeat sequences, such as LINE-1 and Alu, is related to cancer progression and is a regular feature of advanced PCa tumors [102-104]. Altered DNA methylation typically interacts with changes in chromosomal structures, and cooperates with histone modifications in gene silencing. The polycomb group complexes, which are implicated in methylation of histone proteins, are organized in two distinct multiproteins, PRC1 and PRC2. PRC2 is thought to initiate silencing and facilitates the recruitment of PRC1 to maintain stable gene repression[102]. EZH2 histone methyltransferase is part of the PRC2 complex. Overexpression of EZH2 is associated with a higher frequency of promoter hypermethylation and LINE-1 hypomethylation, suggesting a direct link between the PRC2 complex and DNA methylation patterns [105]. Analyses of patterns of epigenetic alteration are providing important insights into the origin of prostate cancer and specific alterations may serve as useful diagnostic and prognostic biomarkers.

1.7 Aberrant DNA Methylation in Prostate Cancer

1.7.1 Advantages associated with methylation biomarkers

DNA methylation changes are far more appealing than other somatic genome alterations for PCa detection, diagnosis, staging, and risk stratification because they appear to occur consistently, arise early, and are potentially reversible[106]. Point mutations could occur throughout the coding region of a given gene, while promoter methylation happens over a
specific region of the gene, greatly simplifying the designing and interpretation of screening tests. In addition, aberrant CpG hypermethylation usually does not occur in normal cells. Therefore, one copy of tumor-derived DNA can be detected with a high degree of specificity against a background of 10,000 unmethylated alleles [7], enabling its application on scarce tumor tissue DNA or on urine and serum samples diluted by excess normal DNA[107]. Finally, epigenetic alterations do not change genome sequence directly, therefore it is reversible and demethylating agents and inhibitors of histone deacetylases (HDIs) are being used in clinical trial [108]. Within the last couple of years, the U.S Food and Drug Administration has approved several new drugs including azacitidine (Vidaza) and decitabine (Dacogen), which function as inhibitors of DNA methyltransferases (DNMTs) to reduce CpG island hypermethylation and reactivate tumor suppressor genes in dividing cancer cells[109].

1.7.2 Diagnostic Potential of DNA Methylation Markers in Prostate Cancer

More than 50 gene loci have been identified to be targets of epigenetic gene silencing in PCas[7]. Aberrant hypermethylation of the GSTP1 promoter is the most well characterized diagnostic marker of PCa. The pi-class glutathione S-transferase gene 1 (GSTP1) is a member of the GST enzyme family that catalyze intracellular detoxification reactions, including the inactivation of electrophilic carcinogens, by conjugating chemically reactive electrophiles to glutathione[110]. Methylation in the regulatory sequences of GSTP1 promoter constitutes the best characterized genomic alteration yet described in PCas. GSTP1 CpG island hypermethylation occurs in 90% of cancers and 70% of precursor high grade prostatic intraepithelial neoplasia (PIN) lesions [111], in contrast to no methylation found in DNA from normal prostate tissues, or from benign prostatic hyperplasia (BPH) tissues[100, 110, 112]. Quantitative GSTP1 methylation reliably discriminates benign from malignant
prostate tissues, and thus serves as a useful diagnostic marker for prostate cancer detection and diagnosis.

The extent of hypermethylation of other genes has also been assessed in PCAs. The CpG islands associated with \(APC, RASSF1a, PTGS2, MDT1, RUNX3, RAR\beta2, CD44, E-cadherin\) etc. are reported to be methylated at high frequencies in primary PCa tumors or PIN lesions, but low or virtually no methylation in normal prostate tissues [113-116]. One example is \(O^6\)-Methylguanine-DNA-Methyltransferase (MGMT) which is a DNA repair gene that functions to remove mutagenic and cytotoxic alkyl groups from \(O^6\)-guanine in DNA. Its promoter was found to be hypermethylated in various cancer types including PCa resulting in loss of function [117, 118].

In addition, many studies have employed a panel of methylation markers to maximize sensitivity and specificity of cancer detection [116, 119-121]. There are several advantages associated with exploiting multiple gene loci to discriminate malignant from benign cells. First of all, the maximum sensitivity a single gene can achieve is only as high as its frequency of hypermethylation[122]; as a result, a combination of multiple epigenetic markers could substantially raise the maximum sensitivity of a molecular test. Secondly, CpG island methylation, in some cases, could occur in noncancerous tissues[122], and a gene panel greatly reduces the chance of detecting non-specific and/or age-related methylation alterations. For example, the panel including \(APC, PTGS2,\) and \(RAR-beta\) showed a sensitivity of 90.8% and specificity of 97.0% for molecular detection of PCa [123].
1.7.3 Prognostic Potential of DNA Methylation Markers in Prostate Cancer

One of the major current challenges in PCa care is to find a reliable marker to differentiate rapidly progressing tumors from slow, indolent ones. A substantial amount of progress has been made in the high-throughput epigenomic screening for the identification of novel prognostic markers that could augment prediction of outcome for PCa patients. Correlations between methylation status and indicators of poor prognosis including high GS, advanced stage, positive surgical margin, early biochemical recurrence and patient mortality have been made for several genes such as CD44, PTGS2, APC, E-cadherin, CDH13, RASSF1A, etc [120, 124-127]. PTGS2 hypermethylation has been reported to correlate with seminal vesicle infiltration, capsular penetration, pathological stage and biochemical recurrence[123]. Hypermethylation of RASSF1A, CDH1, and GSTP1 genes were shown to be significantly more frequent in the high GS group than in the low GS group, and were associated with early PSA recurrence[120]. Recently, Weiss and colleagues reported that PITX2 methylation profile provided prognostic information independent of currently used clinical variables in predicting PSA-free survival [128], and further validated their finding in an independent patient cohort using a customized EpiChip PITX2 microarray [129].

When the methylation profiles of a panel of epigenetic markers are assessed, studies employ variables termed “methylation index”, which is the ratio of methylated genes to the total number of genes analyzed[114, 120, 130], and “M-score”, which is calculated as the sum of the corresponding log hazard ratio coefficients derived from multivariate logistic regression analysis of methylation status of various genes[115, 131], to maximize correlations with clinicopathological variables.
1.7.4 Early Detection of Methylation Alterations in Bodily Fluids

DNA hypermethylation is believed to be an early event in neoplastic progression [132]. DNA from PCa cells are directly shed into the circulation through prostatic ducts and detection of specific methylated biomarkers in serum and urine samples serve as a non-invasive diagnostic that facilitate the detection of curable PCa tumors. Many studies have provided evidence that PCa specific methylation changes can be assayed in bodily fluids. For example, the first attempt to detect methylation changes in PCa patient plasma and serum found 72% of cases exhibited hypermethylation of the \textit{GSTP1} promoter, but no methylation was detected in any of the BPH samples [133, 134]. Later study observed similar results where the frequencies of promoter hypermethylation of \textit{GSTP1, RASSF1A, APC} and \textit{RARb} were significantly higher in PCa patient whole-blood DNA extracts than that of normal controls [135]. Furthermore, one recent study evaluated GSTP1 methylation frequency both in urine collected after prostatic massage and in core needle biopsies from 100 men [136]. It was suggested that detection of GSTP1 methylation in prediagnostic urine exhibited higher specificity compared to that of tissue biopsy [136]. Therefore, the ability to assay methylation changes in blood and urine on diagnostic samples has offered great promise to reduce unnecessary biopsies and improve accuracy of our current clinical diagnostic tools.

1.7.5 Recent Surge in Methylation Detection Techniques

Numerous reports of the implication of DNA methylation in cancer pathogenesis have emphasized the need for accurate, sensitive, reliable and quantitative methods to measure levels of DNA methylation at specific gene loci [137]. Many methylation detection
techniques, including Cobra (combined bisulfite restriction analysis), methylation specific
PCR, and MethyLight, have been developed and refined over the last decade.

Cobra is a quantitative technique that determines DNA methylation levels at specific gene
loci in small amounts of genomic DNA [137]. It is compatible with paraffin sections and can
be applied to large numbers of samples [137]. Methylation levels in the original DNA
samples are measured by the relative amounts of digested and undigested PCR products by
restriction enzymes. However, the Cobra assay is mainly confined to detect methylation in
restriction enzyme digestion sites. This assay is also very time-consuming and labor
intensive. It requires many steps including PCR purification, restriction digestion,
polyacrylamide gel electrophoresis, electroblotting, oligo hybridization and phosphorimager
quantitation [137].

Methylation-specific PCR (MSP) allows for rapid analysis of many samples at multiple gene
loci. MSP is also compatible with paraffin-embedded materials. Nevertheless, MSP cannot
assess the overall level of methylation quantitatively, requires a large amount of DNA, and it
cannot be used in a high throughput way.

Differential hybridization array is a powerful, high throughput method for simultaneously
analyzing the methylation status of hundreds of pre-selected genes. It couples enzymatic
method to array-based analysis [138]. Next-generation sequencing techniques emerged
recently to analyze read-out from restriction enzyme enrichment techniques instead of array
hybridization. It is much more powerful as it allows for allele-specific DNA methylation
analysis. It also avoids hybridization artifacts and does not require an appropriately designed
microarray. However, both techniques are very expensive, and mainly used to survey
genome-wide methylation profiles with high confidence, not suitable for specific gene loci analysis.

MethyLight is a fluorescence-based real-time PCR technique that quantitatively measures methylation status at each designed locus. It is a high throughput assay that is not only highly specific, sensitive and quantitative, but requires very small amounts of DNA and consequently compatible with DNA obtained from microdissected paraffin-embedded tissue samples. Therefore, MethyLight becomes our optimal choice for the study of methylation status at individual biomarker loci.

1.8 CpG Island Methylation and Gene Transcription

1.8.1 Gene Promoter Methylation Suppresses Expression

Promoter hypermethylation has long been recognized to contribute to silencing of tumor suppressor genes. Two mechanisms of DNA methylation-mediated transcriptional repression have been envisaged. In the first mechanism, proteins, usually transcriptional activators, are denied access to their cognate DNA sequence by the methyl groups [139, 140]. The second mechanism involves recruitment of $^5m$CpG-binding domain (MBD) family proteins to maintain chromatin in a transcriptionally inactive state [141, 142]. Inactivation of many genes via the means of promoter methylation has been reported in PCa and might play an important role in PCa development [142-145].

1.8.2 Intragenic Hypermethylation Leads to Increased Gene Transcription

Approximately half of CGIs occur within or in between genes [7, 141]. These CGIs are preferentially susceptible to methylation, but exhibit poor correlation between CpG island
hypermethylation and the silencing of transcription of associated genes [139]. In fact, emerging evidence suggests that methylation of intragenic sequences, within the gene body or at the 3’ end, is associated with increased expression [140, 146]. The mechanistic basis for transcription-coupled methylation remains largely unknown. One possible mechanism is that passage of the transcriptional machinery temporarily disrupts chromatin structure and leads to the transient formation of single-stranded DNA, which serves as favorable substrate for de novo methylation [146-149]. The possible biological significances associated with 3’-end methylation are to suppress antisense transcripts and regulate polyadenylation and/or transcription termination [147].

The concept of “methylation paradox” has been proposed to explain the contrasting downstream effect associated with promoter methylation versus intra-/intergenic methylation [147]. It seems that for genes with a single promoter, a straightforward functional role for promoter CGI methylation in transcriptional repression is supported [140]. In the case where genes are able to generate multiple transcripts by utilizing alternative transcription start sites-associated with alternate promoters, their expression tend to be tissue-specific and do not correspond strictly with the status of promoter methylation. Intragenic methylation could act to prevent transcription initiation from spurious promoter sites [140, 150]. Alternatively, intragenic methylation may repress the expression of antisense non-coding RNA transcription, which would otherwise interfere with the expression of sense transcript [150] (Figure 1.5).
1.8.3 The Concept of CpG Island Shore

Recently, Feinberg and colleagues published two studies reporting that tissue-, cancer-, and reprogramming-specific differential methylation alterations do not occur predominantly within the promoter region or in CpG Islands, rather in sequences within 2kb of islands, termed CpG island shores [151, 152]. CpG island shores have comparatively low CpG density in relation to CpG Islands and are conserved between human and mouse [151, 153]. Another group has also observed similar findings in gliomas [154], where cancer induced methylation occurred in CpG island shores rather than CpG Islands. Methylation alterations in CpG island shores has also been shown to contribute to osteogenic differentiation [155], providing compelling evidence of the importance of CpG island shores. It seems that CpG island shores play key roles in gene transcriptional silencing and serve as epigenetic targets for defining cell fate and cancer development.

Figure 1.5 Schematic representation of CpG island gene association in
a) A simple gene with one CpG associated promoter
b) A more complex gene with alternative promoters (P1-3), multiple intragenic CpG islands (i-v), and an antisense transcript (dashed red arrow)

1.9 Hypothesis, Major Goal and Specific Aims

1.9.1 Hypothesis:

I hypothesize that histologically similar prostate tumors likely express distinct epigenetic profiles therefore leading to different courses of tumor progression.

1.9.2 Specific Aims:

1. My first aim is to determine quantitative methylation levels of two epigenetic markers already implicated in PCa, namely *APC* and *RASSF1A*, using quantitative real-time PCR assay (MethyLight) in a series of 219 primary PCa cases with detailed clinicopathological follow-up data.

2. My second aim is to a) select novel PCa epigenetic markers identified through differential Human CpG island array profiling (previously carried out by another member of our lab) and validate their methylation status in the CpG island regions using EpiTYPER; and b) independently assess the quantitative methylation level of these newly discovered markers using MethyLight assay in the same series of primary PCa cases.

3) My final aim is to establish relationships between epigenetic profiles of genes characterized in Aim 1&2 and patients’ clinicopathological features, in particular, GS, GP, pathological stage and biochemical recurrence.

1.9.3 Major Goal:

Through assessing methylation status of selected epigenetic markers alone or in combination with certain clinicopathological variables, my goal is to evaluate their potential as biomarkers of early diagnosis or prognosis of prostate cancer.
Chapter 2

Promoter Methylation Characterization of Two Known Diagnostic Markers: APC and RASSF1A
2.1 Introduction:

The number of clinically insignificant tumors resulting in potentially unnecessary treatment has dramatically increased because of the widespread use of PSA testing. DNA hypermethylation is a common epigenetic abnormality in PCa and may serve as a molecular based test that could complement the currently existing clinical and histological analyses to stratify patients into diverse risk groups. I performed studies on two known epigenetic markers in PCa: *APC* and *RASSF1A* genes because they are well characterized tumor suppressor genes with recognized diagnostic and prognostic value in prostate cancer.

2.1.1 Background of Adenomatosis polyposis coli (APC) Gene

The *APC* gene consists of 8535 bp spanning 21 exons [146]. The APC protein occurs in multiple forms varying in molecular weight from approximately 90-300kDa and is expressed in specific epithelial and mesenchymal cells of several human tissues. The commonest isoform of APC encodes is 2843 amino acids in length [156]. Exon 15 of the major transcript comprises more than 75% of the coding sequence of *APC* and is the most common target for both germline and somatic mutations. The majority (approximately 95%) of germline mutations in *APC* are nonsense or frameshift mutations that result in a truncated protein product with abnormal function [157]. The *APC* gene has two promoter regions, 1A and 1B [158]. The 1A region of the *APC* promoter has been shown to be heavily methylated in a number of human gastrointestinal tumors, including colorectal, esophageal, gastric, pancreatic and hepatic cancers [159], [160]. There are 31 CpG sites in promoter 1A of the *APC* gene[161]. There is no evidence for such an epigenetic mechanism with the *APC* 1B promoter.
The APC protein consists of numerous domains: an oligomerization domain and an armadillo region in the N-terminus, and a basic domain, binding sites for EB1 and the human disc large (HDLG) protein in the C-terminus. The multiple domains of the APC protein allow it to interact with numerous protein partners (Figure 2.1). APC is an important regulator of the Wnt-signaling pathway through its interaction with β-catenin. β-catenin binds to APC and axin in a complex that promotes GSK3β mediated phosphorylation and subsequent degradation by ubiquitin-mediated proteolysis (Figure 2.2).

**Figure 2.1** The multiple domains and functions of APC

Adapted from *Adenomatous Polyposis Coli (APC): a multi-functional tumor suppressor gene*. *Journal of Cell Science* 2007.120, 3327-3335
2.1.2 Background of Ras Association Domain Family Member 1, Transcript A (RASSF1A) Gene

The RASSF1 gene locus spans about 11,151 bp of the human genome and is comprised of eight exons [119, 120, 126, 162]. The two major promoters of RASSF1 are located in two separate CpG islands [109]. Eight transcripts (RASSF1A-H) are made by alternative promoter
usage and differential splicing of the exons [163] (Figure 2.3). RASSF1A encodes for a protein of 340 amino acids with a molecular mass of 38.8 kDa, and it is ubiquitously expressed. It is a cell cycle regulator, first discovered to be inactivated in lung tumors, and has been implicated in the pathogenesis of a wide spectrum of tumors [109]. Two CpG Islands are associated with the promoter regions of the RASSF1 gene, and the promoter of RASSF1A is located in the first CpG Island.

**Figure 2.3** RASSF1 gene locus and domain structure of the different RASSF1 isoforms. (A) The RASSF1 gene locus is characterized by eight exons (boxed regions) and two different promoters (arrows) with two associated CpG islands (black bars). Black boxes represent coding regions and white boxes are non-coding regions. (B) Schematic representation of the different RASSF1 isoforms.

Adapted from Donninger H, Vos MD, Clark GJ. The RASSF1A tumor suppressor. *J Cell Sci.* 2007 Sep 15;120 (Pt 18):3163-72.
2.1.3 Why Study APC and RASSF1A?

APC hypermethylation has long been established to have potential in PCa diagnosis; it could accurately discriminate benign prostate tissues from BPH and primary tumors [113, 114, 164]. In addition, methylation status of APC consistently showed significant correlation with clinicopathological variables including tumor stage and grade[164]. Hypermethylation of APC was shown to be a significant predictor of time to PCa progression in GS7 patients [119]. Methylation in APC has also been associated with an increased risk of prostate cancer–specific mortality [126, 162]. Finally, tumors with promoter hypermethylation fail to express transcripts of APC, suggesting APC promoter hypermethylation as a mechanism of APC inactivation[165].

Epigenetic inactivation of RASSF1A has also been shown to be a very common event in primary prostate tumors. Aberrant promoter methylation of RASSF1A has been frequently (>70%) detected in several prostate cancer studies, while methylation of RASSF1A has been detected in normal tissues with much lower frequency [109, 113, 166]. A direct correlation between promoter methylation and loss of RASSF1A expression has been shown in many tumors cells[163]. Furthermore, RASSF1A hypermethylation is easily detected in bodily fluids of cancer patients, making it a promising new diagnostic approach to screen putative cancer patients[167]. It has been reported that increased RASSF1A promoter methylation is correlated with advanced tumor grade and stage, although not consistently, and could not predict patient recurrence or survival significantly [120, 162, 164].

However, the majority of published studies have examined gene methylation as a dichotomous variable: any methylation versus no methylation [113, 114, 164, 168]. Depending on the extent of gene promoter methylation, the resultant gene expression levels
and the consequent downstream signaling effects are likely to differ. Thus, quantitative assessment of gene methylation levels may provide better insights into their contribution to prostate carcinogenesis. This point is underscored in a previous study of the best characterized diagnostic methylation marker in PCa, \textit{GSTP1} methylation, in which quantitative \textit{GSTP1} methylation levels were found to significantly correlate with Gleason grade and tumor volume in prostate needle biopsies\cite{101}. In addition, my study employs 219 primary PCa tumors spanning the entire spectrum of primary prostate cancer progression from low grade, prognostically favorable primary prostate tumors to aggressive and highly metastatic ones. Consequently, the alterations in \textit{APC} and \textit{RASSF1A} methylation profiles could be monitored closely at each stage of PCa tumorigenesis. Finally, a unique aspect of my study is that matched normal and multiple tumor foci representing different GPs are collected from the same patients. This provides me an opportunity to identify variations in \textit{APC} and \textit{RASSF1A} methylation levels among individual tumor foci from the same patient indicative of cancer progression. In the current chapter, I systemically assessed the quantitative promoter methylation levels of \textit{APC} and \textit{RASSF1A}, in 219 primary prostatectomy tumors with detailed clinicopathological follow-up data.
2.2 Materials and Methods:

2.2.1 Patient Cohort

A total of 219 formalin-fixed, paraffin-embedded radical prostatectomy patient specimens, diagnosed between 1998 and 2001, were collected at the University Health Network (UHN) in Toronto. All patients who received neo-adjuvant therapy prior to radical prostatectomy were excluded from the study. All patients had consented to tissue collection, banking and utilization in research studies according to the protocols approved by the Research Ethics Board at The University Health Network and Mount Sinai Hospital, Toronto. Patient tissue and clinicopathological data collection, storage and processing were described previously [163]. The clinicopathological characteristics of these 219 patients are listed in Table 2.1. A significant proportion of prostate tumors analyzed in the current study were composed of multiple GPs, and each GP was assayed individually.

2.2.2 DNA Extraction and Bisulfite Modification of Paraffin Embedded Prostate Tissues

The complete set of H&E stained slides complementary to all the paraffin embedded tissue blocks were reviewed by an expert pathologist (Dr. Theo Van der Kwast). Gleason score, tumor stage, and surgical margin status were verified. Cancerous areas containing minimum 80% neoplastic cellularity were marked for different Gleason Patterns. Matched normal tissue containing at least 50% glandular content was also selected for each case where possible. Formalin-fixed paraffin embedded blocks were sectioned into 10-micron sections and superimposed on matching H&E slides and areas of cancer were outlined. The cancerous tissues from circled areas were then manually microdissected and genomic DNA was isolated using the QIAamp DNA mini kit (Qiagen, Mississauga, ON, Canada) using a modified
protocol with extended proteinase K digestion. Briefly, microdissected tissue was digested in 30uL proteinase K at 56°C overnight, followed by an addition of 20uL proteinase K and digestion for one hour at 56°C the following day. Prolonged digestion was used to obtain maximum DNA yield. The Qiagen recommended protocol was then followed for tissue DNA extraction.

Table 2.1 Clinical characteristics of patient cohort

<table>
<thead>
<tr>
<th>Clinical Characteristic</th>
<th>No. Patients (%)</th>
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<tr>
<td>Age</td>
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<tr>
<td>Average</td>
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<tr>
<td>Range</td>
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<td>Pathological Stage</td>
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<tr>
<td>pT2</td>
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<tr>
<td>pT3a</td>
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<tr>
<td>pT3b</td>
<td>19 (8.7)</td>
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<td>pT4</td>
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<td>Gleason Score</td>
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<td>2 (0.9)</td>
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<tr>
<td>5</td>
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<td>52 (23.7)</td>
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<td>Negative</td>
<td>167 (76.3)</td>
</tr>
<tr>
<td>Total Patients</td>
<td>219</td>
</tr>
</tbody>
</table>
Since methylation information is not retained during amplification steps such as PCR, a methylation-dependent modification of the original genomic DNA needs to be performed before any amplification step [107]. Extracted genomic DNA (400ng) was sodium bisulfite treated using the EZ DNA Methylation Gold Kit (Zymo Research Corp, CA, USA) according to manufacturer’s recommended protocol. Treatment of genomic DNA with sodium bisulfite followed by alkaline treatment convert unmethylated cytosines to uracil, while leaving methylated cytosine residues intact [107]. This modification creates methylation-dependent sequence differences in the genomic DNA.

2.2.3 MethyLight Assay

MethyLight is a fluorescence-based real-time PCR technique that measures methylation status at each designed locus (Figure 2.4). It is not only highly specific, sensitive and quantitative, but requires very small amounts of DNA and is consequently compatible with DNA obtained from microdissected paraffin-embedded tissue samples. The assay was performed as previously described [107, 169, 170]. Briefly, a set of PCR primers flanking a dual-labeled fluorogenic hybridization probe are designed specifically for bisulfite converted DNA sequences, representing fully methylated DNA. The probe consists of a 5’ fluorescent reporter dye (6FAM) and a 3’ quencher dye (TAMRA). Perfect annealing between the probe and methylated DNA sequence results in cleavage of the probe by 5’ nuclease activity of Taq polymerase during PCR amplification. This releases the reporter, whose fluorescence can be detected, and the generated fluorescent signal is reflective of the initial DNA template quantity. Partial annealing between the probe and unmethylated DNA sequence only leads to probe displacement, and therefore no florescent signal detection. 20ng of bisulfite-modified DNA was used as a template for real-time PCR. Amplification of AluC4 sequence was
carried out in parallel to control for input DNA. All reactions were run in duplicates in 96 well plates on an ABI 7500 Real-Time PCR system. The percentage of methylated reference (PMR) at a specific locus was calculated by dividing the \textit{GENE: Alu-C4} ratio of a sample by the \textit{GENE: Alu-C4} ratio of commercially available fully methylated DNA and multiplying by 100. Primer and probe sequences used for \textit{APC} and \textit{RASSF1A} were from published sequences [107, 169, 170]: 1) For \textit{APC}: (Forward) 5'-'GAA CCA AAA CGC TCC CCA T -3'; (Reverse) 5'-'TTA TAT GTC GGT TAC GTG CGT TTA TAT -3'; (Probe) 5'FAM-CCC GTC GAA AAC CCG CCG ATT A -BHQ1-3'. 2) For \textit{RASSF1A}: (Forward) 5'-'ATT GAG TTG CGG GAG TTG GT-3'; (Reverse) 5'-'ACA CGC TCC AAC CGA ATA CG -3'; (Probe) 5'FAM-CCC TTC CCA ACG CGC CCA -BHQ1-3'.
2.2.4 Statistical Analysis

The final PMR score of each sample was obtained by averaging results from duplicate runs. If a patient had multiple tumor samples, a final methylation level was assigned by averaging all tumor PMRs of that individual as described previously [169]. We analyzed the prognostic significance of known clinical and pathological characteristics (including GS, stage and surgical margin) in the patient series by constructing Kaplan-Meier curves and by Logrank test. For statistical purposes, PCa samples were divided into four pattern categories: normal, GP2, GP3 and GP4/5, or into three grade categories: GS≤6, GS7, and GS≥8, or into three stage categories: pT2, pT3a, and pT3b/pT4. GP 4 and 5 were grouped together for statistical analysis due to a small number of observations for GP 5 carcinomas (n=8), and pT3b and pT4 were combined for the same reason (n=5 for pT4). PCa samples were also classified into two methylation groups: high methylation (HM) group, which was equal or greater than the third quartile PMR values of the gene of interest, and low methylation (LM) group, which accounted for the rest of the samples [171]. Association between methylation and GS, GP, and pathological stage was examined using the Mann-Whitney U test (for mean) and Pearson Chi-square (for frequency). If any spreadsheet cell had an expected outcome less than 5, then Fisher’s Exact Test was used instead of Pearson Chi-square. Paired t-test was used to assess methylation differences between matched normal and cancerous tissues and between different tumor foci from the same patient. Using a multivariate Cox-regression model comprising clinicopathological and methylation variables, the relative contribution of each variable to biochemical recurrence was assessed. The sensitivity and specificity of quantitative methylation in discriminating cancer versus normal tissue was determined by receiver operator curve (ROC) analysis. For all statistical analyses, a p-value of ≤ 0.05 was
considered significant. All analyses were done using SPSS (Chicago, IL) and R statistical software.
2.3 Results:

2.3.1 Correlation of Methylation Status with Gleason Pattern

I established PMR threshold values using ROC curves that allowed accurate distinction between benign and malignant tissue with maximum sensitivity and specificity (Figure 2.5). The cutoff PMR values were 9.13 for AP
c and 42.82 for RASSF1A. The average methylation level and HM frequency of APC and RASSF1A in prostate adenocarcinomas (stratified by GP), and their matching normals are described in Table 2.2. The average methylation level of both genes were significantly higher in any tumor pattern than that observed in normal (Tables 2.3, p<0.001 for all). The proportion of HM cases of APC and RASSF1A were greater in GP3, GP4/5 and LNM compared to normal (Table 2.3, p<0.001 for all). Substantial increases in HM frequency and average methylation of APC were observed for GP2 versus GP4/5 and GP3 versus GP4/5 comparisons (Table 2.3, Mann-Whitney U p=0.028 and p=0.004, Chi-square p=0.008 and p=0.031). For RASSF1A, both the average methylation and the HM frequency differed between GP2 versus GP3 and GP2 versus GP4/5 (Table 2.3, Mann-Whitney U p=0.001 and p<0.001, Chi-square p=0.006 and p<0.001). In addition, the HM frequency of RASSF1A was significantly different for GP3 versus GP4/5 (Table 2.3, p=0.017), and the mean PMR value of RASSF1A was considerably different when comparing GP4/5 and lymph node metastasis (LNM) tissues (Table 2.3, p=0.024).

Among paired samples, where multiple different patterns came from the same individual, the methylation levels of APC and RASSF1A were significantly higher in tumor than that in normal (Table 2.4, p<0.001), and in GP4/5 compared with GP3 (Table 2.4, p<0.001 for APC,
and \( p = 0.004 \) for \( RASSF1A \). The methylation levels of both genes decreased going from GP4/5 to LNM although the changes were not statistically significant.

Lastly, instead of analyzing all cases within the same GP category as a whole, we stratified GP further according to the GS they were derived from. In this way, we were able to make comparisons between GP3 from GS6 patients and GP3 from GS7 patients, also GP4 from GS7 with GP4 from GS8 cases (Table 2.5). No significant differences were observed for the frequency of methylation of both genes.

### 2.3.2 Correlation of Methylation Status with Gleason Score

The quantitative methylation levels of \( APC \) and \( RASSF1A \) were analyzed in relation to GS. The average methylation level and the proportion of HM cases for each analyzed gene associated with different subgroups are described in Table 2.2. For \( APC \), the average methylation was significantly increased in GS7 PCa compared with GS \( \leq 6 \) PCa (Table 2.3, \( p = 0.018 \)), and both the average methylation and proportion of HM cases were significantly greater in GS \( \geq 8 \) PCa compared with GS \( \leq 6 \) PCa (Table 2.3, Mann-Whitney U \( p = 0.036 \) and Fisher’s Exact \( p = 0.013 \)). On the contrary, for \( RASSF1A \) methylation, no difference was observed when comparing the three GS groups (Table 2.3).
Figure 2.5 Optimal sensitivity and specificity determined by receiver operating characteristics (ROC) curves for the two hypermethylation gene loci (A) APC methylation, and (B) RASSF1A methylation. Maximum specificity and sensitivity achieved and the optimal cut-off value for each gene are shown.

Area under the Curve (AUC): 0.919; Sensitivity: 0.824; Specificity: 0.952

Area under the Curve (AUC): 0.937; Sensitivity: 0.920; Specificity: 0.856
Table 2.2 *APC* and *RASSF1A* methylation profiles stratified according to Stage, Gleason Score and Gleason Pattern.

<table>
<thead>
<tr>
<th>Stage</th>
<th>APC Methylation Status</th>
<th>RASSF1A Methylation Status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average PMR</td>
<td>Percentage of HM</td>
</tr>
<tr>
<td>pT2</td>
<td>32.93</td>
<td>18.4% (26/141)</td>
</tr>
<tr>
<td>pT3a</td>
<td>47.94</td>
<td>37.0% (20/54)</td>
</tr>
<tr>
<td>pT3b/4</td>
<td>44.14</td>
<td>37.5% (9/24)</td>
</tr>
<tr>
<td>Gleason Score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤6</td>
<td>32.81</td>
<td>17.6% (18/102)</td>
</tr>
<tr>
<td>=7</td>
<td>41.46</td>
<td>28.6% (28/98)</td>
</tr>
<tr>
<td>≥8</td>
<td>46.43</td>
<td>47.4% (9/19)</td>
</tr>
<tr>
<td>Gleason Pattern</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>2.26</td>
<td>0% (0/62)</td>
</tr>
<tr>
<td>2</td>
<td>29.89</td>
<td>11.1% (3/27)</td>
</tr>
<tr>
<td>3</td>
<td>34.69</td>
<td>26.1% (47/180)</td>
</tr>
<tr>
<td>4/5</td>
<td>45.40</td>
<td>37.4% (52/139)</td>
</tr>
<tr>
<td>LNM</td>
<td>37.30</td>
<td>26.7% (4/15)</td>
</tr>
</tbody>
</table>

LNM stands for lymph node metastasis
HM stands for high methylation and is defined as equal or greater than the 3rd quartile PMR value of the gene of interest
Table 2.3 P-values for APC, TGFβ2, and RASSF1A methylation stratified by clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>APC Methylation</th>
<th>RASSF1A Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mann-Whitney U</td>
<td>Chi-square</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT2 vs. pT3a</td>
<td>&lt;0.001</td>
<td>0.006</td>
</tr>
<tr>
<td>pT2 vs. pT3b/pT4</td>
<td>0.074</td>
<td>0.035</td>
</tr>
<tr>
<td>pT3a vs. pT3b/pT4</td>
<td>0.661</td>
<td>0.969</td>
</tr>
<tr>
<td>Gleason Score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤6 vs. 7</td>
<td>0.018</td>
<td>0.066</td>
</tr>
<tr>
<td>≤6 vs. ≥8</td>
<td>0.036</td>
<td>0.013*</td>
</tr>
<tr>
<td>7 vs. ≥8</td>
<td>0.423</td>
<td>0.107</td>
</tr>
<tr>
<td>Gleason Pattern</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal vs. 2</td>
<td>&lt;0.001</td>
<td>0.026*</td>
</tr>
<tr>
<td>Normal vs. 3</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Normal vs. 4/5</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Normal vs. LNM</td>
<td>&lt;0.001</td>
<td>0.001*</td>
</tr>
<tr>
<td>2 vs. 3</td>
<td>0.496</td>
<td>0.089</td>
</tr>
<tr>
<td>2 vs. 4/5</td>
<td>0.028</td>
<td>0.008</td>
</tr>
<tr>
<td>2 vs. LNM</td>
<td>0.510</td>
<td>0.440*</td>
</tr>
<tr>
<td>3 vs. 4/5</td>
<td>0.004</td>
<td>0.031</td>
</tr>
<tr>
<td>3 vs. LNM</td>
<td>0.590</td>
<td>1.000*</td>
</tr>
<tr>
<td>4/5 vs. LNM</td>
<td>0.440</td>
<td>0.540</td>
</tr>
</tbody>
</table>

* Fisher’s Exact Test is used instead of Chi-square Test

LNM stands for lymph node metastasis

Table 2.4 Paired t-test comparisons of APC and RASSF1A methylation levels according to Gleason patterns on a patient by patient basis

<table>
<thead>
<tr>
<th>Comparison</th>
<th>APC Methylation</th>
<th>RASSF1A Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average Difference</td>
<td>p-value</td>
</tr>
<tr>
<td>Cancer vs. Normal</td>
<td>34.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2 vs. Normal</td>
<td>14.5</td>
<td>0.42</td>
</tr>
<tr>
<td>3 vs. 2</td>
<td>4.0</td>
<td>0.67</td>
</tr>
<tr>
<td>4/5 vs. 3</td>
<td>10.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LNM vs. 4/5</td>
<td>-18.5</td>
<td>0.052</td>
</tr>
</tbody>
</table>

LNM stands for lymph node metastasis
Table 2.5 Comparisons of the same Gleason pattern cases derived from different Gleason score categories A) Detailed methylation average and HM frequency B) p-values associated with comparisons

A.

<table>
<thead>
<tr>
<th>APC Methylation</th>
<th>RASSF1A Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>Percentage of HM Cases</td>
</tr>
<tr>
<td>GP3 (GS6)</td>
<td>33.67</td>
</tr>
<tr>
<td>GP3 (GS7)</td>
<td>36.59</td>
</tr>
<tr>
<td>GP4 (GS7)</td>
<td>79.07</td>
</tr>
<tr>
<td>GP4 (GS8)</td>
<td>84</td>
</tr>
</tbody>
</table>

HM stands for high methylation and is defined as equal or greater than the 3rd quartile PMR value of the gene of interest

B.

<table>
<thead>
<tr>
<th>APC</th>
<th>P (Mann-Whitney U)</th>
<th>P (Chi-square)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP3(GS6) vs. GP3 (GS7)</td>
<td>0.480</td>
<td>0.430</td>
</tr>
<tr>
<td>GP4(GS7) vs. GP4 (GS8)</td>
<td>0.600</td>
<td>0.980</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RASSF1A</th>
<th>P (Mann-Whitney U)</th>
<th>P (Chi-square)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP3(GS6) vs. GP3 (GS7)</td>
<td>0.023</td>
<td>0.420</td>
</tr>
<tr>
<td>GP4(GS7) vs. GP4 (GS8)</td>
<td>0.430</td>
<td>0.260</td>
</tr>
</tbody>
</table>

2.3.3 Correlation of Methylation Status with Pathological Stage

I also investigated the relationship between quantitative methylation level and pathological stage. Average PMR values and frequency of HM are shown in Table 2.2. For APC methylation, I observed considerable differences in the average PMR between pT2 versus pT3a (Table 2.3, p<0.001). The frequency of APC HM methylation was shown to differ significantly when comparing pT2 versus pT3a and pT2 versus pT3b/pT4 PCa cases (Table 2.3, p=0.006 and p=0.035 respectively). However, no considerable difference was observed for RASSF1A methylation when comparing the three pathological stages (Table 2.3).
2.3.4 Promoter Methylation and Biochemical Recurrence

I next analyzed the prognostic significance associated with \textit{APC} and \textit{RASSF1A} promoter methylation. Univariate log-rank analysis demonstrated that hypermethylation of \textit{APC} was significantly correlated with higher biochemical recurrence rate (Figure 2.6, panel A, \(p=0.028\)). However, such an association was not observed for \textit{RASSF1A} promoter methylation levels (Figure 2.6, panel B, \(p=0.556\)). Next, I performed Kaplan-Meier curves after stratification for GS and pathological stage. \textit{APC} was a significant predictor of biochemical recurrence in pT2 stage patients (Figure 2.7, \(p=0.028\)). Multivariate analyses comprising of five variables (methylation category of gene of interest, GS, stage, surgical margins and age) were performed to test the contribution of each predictor to biochemical recurrence in the presence of other variables. GS, pathological stage and surgical margin status were all significant predictors, but neither \textit{APC} nor \textit{RASSF1A} was an independent predictor of biochemical recurrence (Tables 2.6-2.7). When only pT2 stage patients were examined, APC methylation could significantly predict biochemical recurrence in the presence of GS, surgical margin status and age (Table 2.8).
Figure 2.6 Kaplan-Meier curves of biochemical recurrence for (A) $APC$ methylation status, and (B) $RASSF1A$ methylation status

The blue line indicates PCa cases with low methylation, and the green line indicates PCa cases with high methylation for each gene of interest respectively. HM stands for high methylation and is defined as equal or greater than the 3rd quartile PMR value of the gene of interest, LM represents the rest of the patient cohort.
**Figure 2.7** Kaplan-Meier curves of biochemical recurrence for *APC* methylation status on pathological stage 2 patients.

The blue line indicates PCa cases with low methylation, and the green line indicates PCa cases with high methylation for each gene of interest respectively. HM stands for high methylation and is defined as equal or greater than the 3rd quartile PMR value of the gene of interest, LM represents the rest of the patient cohort.
### Table 2.6 Multivariate Analysis with APC methylation status

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td><em><em>Gleason Score (GS≤6</em>)</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>=7</td>
<td>1.39</td>
<td>0.74</td>
<td>2.62</td>
</tr>
<tr>
<td>≥8</td>
<td>8.23</td>
<td>3.22</td>
<td>21.08</td>
</tr>
<tr>
<td><em><em>Pathological Stage (pT2</em>)</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT3a</td>
<td>3.61</td>
<td>1.85</td>
<td>7.02</td>
</tr>
<tr>
<td>pT3b/pT4</td>
<td>4.09</td>
<td>1.91</td>
<td>8.74</td>
</tr>
<tr>
<td><strong>Surgical Margin Status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.18</td>
<td>0.73</td>
<td>1.92</td>
</tr>
<tr>
<td><strong>APC Methylation Status</strong></td>
<td>2.22</td>
<td>0.78</td>
<td>6.32</td>
</tr>
</tbody>
</table>

*GS≤6 and pT2 represent the baselines
The overall p-value associated with each clinicopathological variable is obtained by comparing the rest of the patient cohort with baseline group

### Table 2.7 Multivariate Analysis with RASSF1A methylation status

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td><em><em>Gleason Score (GS≤6</em>)</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>=7</td>
<td>1.43</td>
<td>0.83</td>
<td>2.48</td>
</tr>
<tr>
<td>≥8</td>
<td>5.48</td>
<td>2.55</td>
<td>11.78</td>
</tr>
<tr>
<td><em><em>Pathological Stage (pT2</em>)</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT3a</td>
<td>2.66</td>
<td>1.54</td>
<td>4.59</td>
</tr>
<tr>
<td>pT3b/pT4</td>
<td>3.22</td>
<td>1.73</td>
<td>6.01</td>
</tr>
<tr>
<td><strong>Surgical Margin Status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.23</td>
<td>0.77</td>
<td>1.96</td>
</tr>
<tr>
<td><strong>RASSF1A Methylation Status</strong></td>
<td>0.74</td>
<td>0.42</td>
<td>1.29</td>
</tr>
</tbody>
</table>

*GS≤6 and pT2 represent the baselines
The overall p-value associated with each clinicopathological variable is obtained by comparing the rest of the patient cohort with baseline group
Table 2.8 Multivariate Analysis with APC methylation status on pT2 stage patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td><em><em>Gleason Score (GS≤6</em>)</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>=7</td>
<td>0.937</td>
<td>0.440</td>
<td>1.996</td>
</tr>
<tr>
<td>≥8</td>
<td>1.823</td>
<td>0.510</td>
<td>6.531</td>
</tr>
<tr>
<td><strong>Surgical Margin Status</strong></td>
<td>2.287</td>
<td>1.073</td>
<td>4.874</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>1.397</td>
<td>0.684</td>
<td>2.852</td>
</tr>
<tr>
<td><strong>APC Methylation Status</strong></td>
<td>2.174</td>
<td>1.044</td>
<td>4.530</td>
</tr>
</tbody>
</table>

*GS≤6 represents the baseline
The overall p-value associated with each clinicopathological variable is obtained by comparing the rest of the patient cohort with baseline group
2.4 Discussion:

In this chapter, I systematically investigated quantitave methylation levels of \textit{APC} and \textit{RASSF1A}, and examined their relationships with clinicopathological features of primary prostate adenocarcinoma. Methylation of \textit{APC} and \textit{RASSF1A} demonstrated great diagnostic potential, since both \textit{APC} and \textit{RASSF1A} methylation levels increased significantly from normal to tumor, and these results are consistent with observations reported earlier [113, 114, 164, 168].

I observed a significant increase in \textit{APC} promoter methylation level going from GP3 (single and separate gland) to GP4 (fused and poorly defined gland), from GS \leq 6 (low grade) to GS7 (intermediate grade) groups, and from pathological stage pT2 (confined to prostate) to pT3a (extends beyond prostate capsule) in our series of archival prostate specimens. All three pathological transitions are indicative of progression from relatively indolent, slow-growing tumors to intermediate stage tumors with the potential to invade. I also analyzed \textit{APC} methylation data using a Yes/No methylation cutoff as determined by ROC curve to be 9.15 (data not shown). The significant change in methylation frequency I observed for GP3 vs. GP4/5 and GS6 vs. GS8 comparisons with quantitative analysis were lost when a Yes/No methylation cutoff was used. It seems that quantitative assessment of gene methylation levels provides better insights into their contribution to prostate carcinogenesis. The extent of gene promoter methylation could greatly influence the resultant gene expression levels and the consequent downstream signaling effects.

Deletion of \textit{APC} gene alone in the prostates of mice has been reported to elevate $\beta$-catenin expression and induce prostate tumor formation by 7 months [172]. The above evidence
implies a causative role of \( APC \) inactivation in PCa, rather than a bystander effect. Increased level of promoter hypermethylation of \( APC \) is proposed to result in decreased APC protein expression and accumulation of \( \beta \)-catenin in both cytoplasm and nucleus, therefore promoting PCa proliferation and progression by activating \( \beta \)-catenin downstream signalling pathways [173]. Taken together, these observations suggest that \( APC \) inactivation facilitates PCa tumor progression and that \( APC \) methylation level is likely to be elevated in biologically more aggressive tumors.

Steady increase in \( RASSF1A \) methylation levels was observed moving from the well-differentiated GP2 to the poorly differentiated GP5, despite similar profiles of \( RASSF1A \) methylation across pathological stage and GS groups. The majority of epigenetic markers discovered to date, including \( GSTP1 \) and \( APC \), had minimal to no methylation in benign tissues, while \( RASSF1A \) promoter methylation is frequently detected in benign tissues surrounding the cancerous areas. It is possible that certain hypermethylation changes occur as a “field effect” which may predispose to or accompany the development of actual carcinomas [102, 174]. Alternatively, hypermethylation of \( RASSF1A \) could happen sporadically in normal cells and doesn’t have a detrimental effect on cell survival. In line with the previous view, \( RASSF1A \) specific knockout mice had been shown to develop various tumor types including lung adenomas, lymphomas, and breast adenocarcinoma, but no prostate tumor was detected [175]. The above evidence suggests that inactivation of \( RASSF1A \) alone is not sufficient to induce PCa formation. Although \( RASSF1A \) has been reported to discriminate normal from cancerous tissues with high sensitivity and specificity, its role in PCa progression remains controversial. Inconsistent results have been reported in exploring association of \( RASSF1A \) methylation level with either GS or stage [113, 120, 162, 164, 166].
Our study has demonstrated high sensitivity and specificity associated with \textit{RASSF1A} for discriminating cancerous from normal tissue. It might be more suitable to assess \textit{RASSF1A} methylation level as a dichotomous variable, and explore its diagnostic potential.

For the majority of prostatectomies included in our study, I was able to collect matched normal and multiple tumor foci from the same patient. Pair-wise comparisons between multiple patterns provided me the unique opportunity to identify differences in methylation levels among individual tumor foci indicative of progression. For matched GP comparisons, both \textit{APC} and \textit{RASSF1A} methylation levels increased significantly from normal to tumor, and also from GP3 to GP4. This indicated that the general correlation between methylation level and Gleason Pattern also held true on an individual basis.

I observed no quantitative difference in methylation levels of both genes when GP3 from GS6 was compared with GP3 from GS7, and GP4 from GS7 was compared with GP4 from GS8 (Table 2.5, panel B), while GP3 from GS7 differed in methylation level than GP4 from GS7 (Table 2.4). It seems that aberrant hypermethylation precedes changes in prostate glandular architecture, rather than following it. This is supported by evidence suggesting locus-specific aberrant promoter hypermethylation is associated with early stage cancer development [102].

I was initially surprised to observe that the promoter methylation levels of \textit{APC} and \textit{RASSF1A} remained approximately the same or experienced a slight drop going from GP4&5 to lymph node metastasis since a higher malignant potential was expected in lymph node metastasis. Previous studies looking at both prostate and breast cancer metastases also found similar results for the methylation profiles of several genes, including \textit{APC} and \textit{RASSF1A},
between primary tumors and their paired metastases[116, 176]. One possible explanation is that during the initial expansion and differentiation stage of the primary prostate tumors, cells harboring the most densely methylated alleles invade surrounding tissues and are selected for. However, after dissemination, metastatic deposits need to acquire alternative epigenetic and expression patterns in order to maintain and survive in a new microenvironment. Epigenetic inactivation is likely to be dynamic and subject to microenvironmetal influences during malignant progression [177]. An alternative theory was proposed by Yegnasubramanian et al that DNA hypomethylation changes could arise late in prostate cancer progression, resulting in significant decreased methylation level at the stage of metastatic disease [104]. It is possible that global hypomethylation leads to reactivation of a network of stem cell transcription factors, which are expressed in early embryonic development, but become downregulated in adult tissues through promoter hypermethylation [178]. A small fraction of androgen-independent stem cells present in PCa tumors could undergo clonal expansion upon gene reactivation and result in the emergence of late-stage, hormone refractory PCa [42, 43].

For PCa cases with HM of APC, there was a significant trend to a higher probability of biochemical recurrence in univariate analysis. This provides some interesting potential insights into underlying biological mechanisms. One possibility is that patients with apparently organ-confined cancer at the time of diagnosis may actually possess metastatic cells that are likely predicted by high APC methylation. However, APC failed to independently predict biochemical recurrence in multivariate analysis. Cancer progression course and aggressiveness are expected to be better recapitulated when taking into account the behavior of a combination of markers, instead of any single marker. Accordingly, I
selected novel PCa epigenetic markers identified through differential Human CpG island array profiling (previously carried out by another member of our lab) and independently validated quantitative methylation levels of these newly discovered markers in the series of 219 primary PCa cases. I anticipated that methylation profiles of a panel of epigenetic markers combined with certain clinicopathological variables may help to predict more accurately which PCas are at risk of rapidly progressing.
Chapter 3

Selection and Subsequent Validation of Two Novel Epigenetic Markers: \textit{CYP26A1} and \textit{TBX15}
3.1 Introduction:

Previous studies have consistently demonstrated that employing a combined methylation status of several genes might have more discriminatory power to PCa diagnosis and/or prognosis [113-115, 168, 179, 180]. Therefore, in addition to APC and RASSF1A, I selected 2 additional novel methylation markers in an attempt to establish a small panel of prognostic biomarkers to better predict patient risks and outcome.

In our lab, a genome-wide differential hybridization CpG island array of GS 6-8 PCa cases was previously performed by Dr. Vaijayanti Pethe and Ken Kron [181]. I proposed that selecting candidate genes that are differentially methylated for patients with various GS or biochemical recurrent rates from this array would yield informative prognostic markers. The methylation status of these candidate markers could be analyzed in single CG dinucleotide resolution, and further validated in independent patient cohorts to identify patients that harbor aggressive, rapidly progressing PCa. In this chapter, I chose and validated 2 DNA methylation markers, CYP26A1 and TBX15, and established relationships between their methylation profiles with patient clinicopathological follow-up data. A schematic diagram of the selection process is briefly described in Figure 3.1.
**Figure 3.1** Overview of the selection process for novel hypermethylated genes in prostate cancer.

BCH Recurrence refers to biochemical recurrence
3.1.1 Gene Structure of Cytochrome P450, Family 26, Subfamily A, Polypeptide 1 (CYP26A1)

CYP26A1 maps to human chromosome 10q23-q24, and belongs to the CYP26 superfamily, which has three members A1, B1 and C1. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. They share extensive sequence similarity, but each catalyzes different derivatives of Vitamin A. CYP26A1 enzyme is involved in metabolic inactivation of retinoids, including all-trans-retinoic acid (RA), with both 4-hydroxylation and 18-hydroxylation activities [182].

3.1.2 The Involvement of CYP26A1 in Cancer

CYP26A1 expression level has been implicated to promote cancer development and progression. It is shown to be highly expressed in primary breast cancers, and promotes anchorage-independent growth of cancer cells[182]. Another study showed that positive expression of CYP26A1 was associated with poor breast cancer survival using tissue microarray [183]. The expression level of CYP26A1 is reported to be higher in primary ovarian cancer as compared to that in normal ovary[184]. In Barrett's associated esophageal adenocarcinoma, the expression level of CYP26A1 is elevated. The role of CYP26A1 has also been studied in a mouse model of prostate cancer. However, no significant changes in CYP26A1 mRNA levels was found in TRAMP mice as compared to the non-transgenic littermate controls[185]. Interestingly, one group demonstrated contradictory finding that associated CYP26A1 overexpression with favorable neuroblastoma tumors [186]. CYP26A1 transcription is shown to be epigenetically regulated [187]; however the exact mechanism
remains unknown. This study was undertaken to investigate the contribution of promoter DNA methylation in regulating CYP26A1 transcription using three prostate cancer cell lines 22RV1, DU-145, and PC-3. Based on the past observations of CYP26A1’s involvement in cancer development, I expect CYP26A1 promoter methylation to exhibit diagnostic and prognostic potential in PCa.

3.1.3 Background of T-box 15 (TBX15)

TBX15 maps to human chromosome 1p13, and currently there have been five protein-coding alternative spliced transcripts identified. It is a member of the T-box gene family that functions as transcriptional activators or repressors[188]. The precise role of TBX15 has not yet been characterized in cancer. TBX15 is shown to be a target gene of the homeodomain transcription factor PITX2, which facilitates the recruitment of coactivators to the TBX15 promoter [189]. Many studies have illustrated the importance of TBX15 in embryonic development processes that include mesoderm formation, patterning and organogenesis. For example, mutations in TBX15 cause a complex cranial, cervical, auricular and skeletal malformation syndrome with scapular and pelvic hypoplasia, known as Cousin Syndrome [190], which recapitulates the skeletal malformation phenotype displayed by TBX15-deficient mice [191]. TBX15 expression level has not been demonstrated previously to be subject to epigenetic regulation, and the current study was undertaken to examine the extent of TBX15 promoter methylation in PCa progression.
3.2 Materials and Methods:

3.2.1 Differential Methylation Hybridization and Human CpG Island Array Profiling

Agilent CpG island arrays coupled with differential methylation hybridization (DMH) were performed previously by other members of our lab, Dr. Vaijayanti Pethe and Ken Kron, on a separate series of fresh-frozen prostate tumors for high-throughput analysis of DNA methylation (n=10 for GS6 cases, n=19 for GS7 cases, n=10 for GS8 cases, and n=5 for normals) [175]. Briefly, isolated genomic DNA was enriched for methylation signals and amplified for microarray hybridization. To identity genes with the most differentially methylated profiles, three comparisons 1) GS6 vs. GS8, 2) GS7 vs. GS8, and 3) Biochemical Recurrence vs. No Biochemical Recurrence, were performed on generated microarray data using various statistical models, including t-test, modified t-test and quantile regression.

3.2.2 MassARRAY EpiTYPER Analysis

Quantitative analysis of CpG dinucleotide methylation was performed using a mass spectrometry approach as available by MassARRAY EpiTYPER analysis (Sequenom). EpiTYPER analysis is a MALDI TOF mass spectrometry based method that provides a quantitative view of CpG dinucleotide methylation to single or multiple dinucleotide resolution. Two fresh-frozen PCa tissues, one case that showed enrichment of methylated CpG dinucleotides and one case that lacked methylation signal from the CpG island array, together with a human prostate cell line DU-145 were submitted for EpiTYPER analysis for each gene. DNA is first bisulfite modified, tagged with a T7 promoter, and transcribed into RNA. This is then cleaved with RNase A and cleavage products of different mass can be resolved by the MS instrument. Analysis is performed by the Analytical Genetics
Technology Centre (AGTC), Princess Margaret Hospital, Toronto, Ontario. Regions analyzed by EpiTYPER correspond to those that showed an enriched signal in the CpG island array results.

### 3.2.3 Cell lines and culture conditions

The prostate cancer cell lines 22RV1, DU-145 and PC-3 were obtained from Dr. Diamandis’s lab in Samuel Lunenfeld Research Institute, Mount Sinai Hospital. 22RV1 was cultured in RPMI-1640 medium (Media Prep SLRI), DU145 in MEM medium (Media Prep SLRI), and PC-3 in F12 medium (Invitrogen, Toronto, ON, Canada) in a 37°C humidified incubator with 5% CO2. 10% fetal bovine serum was supplied to each media to boost growth.

### 3.2.4 5-aza-2’-deoxycytidine treatment followed by DNA and RNA extraction

A 250 µg/ml stock solution of 5-aza-2’-deoxycytidine (Sigma-Aldrich, Oakville, ON, Canada) was prepared in water and kept at −80°C until use. Cells were seeded on 100mm dishes and allowed to attach over 24h. Following that, cells were treated with a final concentration of 2µg/ml 5-aza-2’-deoxycytidine for 48h. At every 24h interval, fresh medium containing 5-AdC was added. The treated cells were then washed once with phosphate-buffered saline (PBS) and allowed to grow in drug-free medium for 48h before they were harvested for DNA and RNA extraction. For control plates, cells were maintained in drug-free medium for 48h, washed and were grown in drug-free medium for another 48h. Both control and 5-AdC treatment plates were prepared in triplicates. Cells were disrupted by adding 600µl RLT plus buffer from AllPrep DNA/RNA kit (Qiagen, Mississauga, ON, Canada). Genomic DNA and total RNA were purified simultaneously following the Qiagen recommended protocol.
3.2.5 Real-time RT-PCR

Five micrograms of total RNA was reverse transcribed using a SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, United States) with random hexamers and oligo dTs in 20µl of total reaction solution, and diluted into 500µl in DNAse/RNase free water. The primers used for CYP26A1 were from published sequences [181]: (Forward) 5’-TAA ATG GAT ACC AGA TTC CCA AGG-3’; (Reverse) 5’-CTT CCT TGT TGG TGA AGA TCT CTG-3’. 2µl of RT cDNA was amplified using the Applied Biosystems 7500 Real-Time PCR System with SYBR Green PCR Master Mix (Applied Biosystems). PCR consisted of 95°C for 10 min, 50 cycles at 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. Each reaction was performed in triplicates. To control for DNA input, amplification of β-actin sequence was carried out in parallel. The final CYP26A1 expression level for each sample was calculated through dividing the expression of CYP26A1 by the expression of β-actin.

Assessing the implication of aberrant promoter methylation on the expression level of CYP26A1 was suggested by my committee members because past studies reported contradictory findings on the biological role of CYP26A1 in cancer.

3.2.6 MethyLight Assay and Statistical Analyses

From 219 prostatectomy cases, genomic DNA was isolated, bisulfite treated and subsequently used for MethyLight assay as described in Chapter 2. Primer and probe sequences used for CYP26A1 and TBX15 were self-designed based on EpiTYPER results: 1) For CYP26A1: (Forward) 5’- TTG TAG AGA TTC GAC GTA CGC GG -3’; (Reverse) 5’- AAA ACC TTC CGT CAA ACA TCC TCT ACG -3’; (Probe) 5’FAM- ACG CCC ACG TAC CCG CTT CCT TAC -BHQ1-3’. 2) For TBX15: (Forward) 5’- GCG GTT TTG
TAA GTA TAT TGT TGC G -3’; (Reverse) 5’- ACT CCG AAT AAA ACA AAA ACT
AAA ATC CG-3’; (Probe) 5’- FAM- CAA ATA ACG CCG CCG AAC GCC T -BHQ1-3’.
A percentage of methylated reference (PMR) score was calculated for each specific gene
locus by dividing the GENE: Alu-C4 ratio of a sample by the GENE: Alu-C4 ratio of
commercially available fully methylated DNA and multiplying by 100. Statistical analyses
on the correlations between gene methylation profiles and clinicopathological variables were
performed as described in Chapter 2.

Epigenetic markers that demonstrated prognostic significance: APC and TBX15, were
grouped and tested for their ability to predict biochemical recurrence using both Kaplan-
Meier curves and Cox multivariate regression model. Patients were divided into three
categories according to their methylation status of APC and TBX15: 1) LM of both APC and
TBX15, 2) HM of either APC or TBX15, 3) HM of both APC and TBX15 (high methylation
(HM) group, which was equal or greater than the third quartile PMR values of the gene of
interest, and low methylation (LM) group, which accounted for the rest of the samples, as
explained in Chapter 2).
3.3 Results:

3.3.1 Candidate Biomarker Selection and Validation using MassARRAY EpiTYPER

A list of genes that exhibited the most different methylation profiles was generated according to the statistically significant p-values from the three comparisons 1) GS6 vs. GS7, 2) GS7 vs. GS8, and 3) Biochemical Recurrence vs. No Biochemical Recurrence performed on microarray data. Potential epigenetic markers of PCa CYP26A1 and TBX15 were selected based on the biological function of the gene, involvement in PCa and statistical significance from CpG microarray results. CY26A1 ranked 14 based on log fold change of the comparison between Biochemical Recurrence vs. No Biochemical Recurrence, while TBX15 ranked 27 for Biochemical Recurrence log fold change and ranked 170 for GS7 vs. GS8 comparison. Next, we validated the methylation patterns of two candidate biomarkers using the EpiTYPER assay. Data obtained from EpiTYPER analyses confirmed the enrichment of methylation profiles of CYP26A1 and TBX15 that were evident from the microarrays. MethyLight primers and probes for CYP26A1 and TBX15 were designed to approximate the regions showing the most differential methylation pattern, and examined 10 possible methylation sites (CpG dinucleotides) in total. Overviews of the three analyses performed: Agilent CpG island array, EpiTYPER and MethyLight primers and probe design on candidate gene markers CYP26A1 and TBX15 are illustrated in Figure 3.2-3.3.
**Figure 3.2** The analysis of \textit{CYP26A1} promoter methylation status in the search for novel prostate cancer diagnostic and prognostic markers

\textbf{Agilent Human CpG Island microarrays}

The color-coded boxes represented cases selected for EpiTYPER analysis.

\textbf{EpiTYPER}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{CpG Number} & \textbf{GS7A} & \textbf{GS7B} \\
\hline
1 & & \\
2 & & \\
3 & & \\
4 & & \\
5 & & \\
6 & & \\
7 & & \\
8 & & \\
9 & & \\
10 & & \\
11 & & \\
12 & & \\
13 & & \\
14 & & \\
15 & & \\
16 & & \\
17 & & \\
18 & & \\
19 & & \\
20 & & \\
\hline
\end{tabular}
\end{table}

\textbf{MethyLight Primer and Probe Design:}

\begin{verbatim}
GTGCTGCTTGGCTTCCCTTCCCGAGCCAAATCCAGGGAGGCTGATGGGGGAAGGCCGAGC
GGTAGGGGTGCGAGGAGTGGGATATTGCAACTCGGGGACATTGCAGAGACGCACGC
GGGACCTGCCACCCTTGTAAGGAAGCGGGCACGCCGTGGCAGGCA
TTTGGGCCATAGAAAAAAGACCCAGAGAATGCTGACGGCCCTGGCAGCTGGCGTC
TGCTTGCTTGCTTGCTTGCTTGCTTGCTTGCTTGCTTGCTTGCTTGCTTGCTTGCT
AGGCTGAAAGGGCTCAAAGGTACCTGCTGGGAAACTAGCAACTGTGGGCACTTCC
CTTTAAAGAAGGCATTTATTCTTG
\end{verbatim}
**Figure 3.3** The analysis of \(TBX15\) promoter methylation status in the search for novel prostate cancer diagnostic and prognostic markers

**Agilent Human CpG Island microarrays**

The color-coded boxes represented cases selected for EpiTYPER analysis.

**EpiTYPER**

**MethyLight Primer and Probe Design:**

- **GCCTCGGAGGAGCCTGGCTCTCGAGCCTGCTCTCCCCCGCCCTCTCCAGAGCAACAGAG**
- **GAACTTTCGAGCTAGGATCGGCTGGAACAGCTGCTCCGGCTGCGGCCTTGCAAGTATATT**
- **CCTTGCAAGTATATTTCAGGCGCTCGGCGGCGCCATCTGCGCTCGGACCTCAG**
- **CCCCTGCTCCACCCGGAGTCCTCAGCTAGGGCTTGGGCCTTTCCCGCCAAAATCTTCTGC**
- **TAAGTGCTGCCGAAGCCGGGTCTCCCGCGGAGCGAAATAATGTAAACCTTCTGTGGAGGG**
- **ATTTCCTCCTCCAGAAACTTCGGGCCGGGCCACACAGCAGCTGCGTTCCAGAGGCCTGGG**
3.3.2 Correlation of CYP26A1 promoter methylation status with mRNA expression in 
human prostate cancer cell lines

*CYP26A1* promoter region was endogenously methylated in three well characterized PCa cell 
lines, 22RV1, DU-145, and PC-3 with methylation PMRs of 44.8, 67.5, and 66.9 respectively 
(represented in Figure 3.4). To investigate whether hypermethylation of the *CYP26A1* 
promoter region led to transcriptional silencing of this gene, I examined the effects of the 
demethylating agent 5-aza-2’-deoxycytidine in those three PCa cell lines. 
The average promoter methylation level of *CYP26A1* was significantly reduced in cells 
undergone demethylating treatment (p<0.001 for 22RV1, p=0.001 for DU-145, and p=0.023 
for PC-3, Figure 3.4), with post-treatment PMR values of 25.9 for 22RV1, 34.7 for DU-145, 
and 43.7 for PC-3. I attempted to further reduce *CYP26A1* promoter methylation levels with 
longer incubation time (96hrs instead of 48hrs) and/or higher 5-aza-2’-deoxycytidine 
concentration (4µg/ml instead of 2µg/ml), but failed to achieve more than 50% reduction 
(data not shown). Next, I performed real-time RT-PCR on *CYP26A1* to examine the 
functional implication of promoter methylation. All three cell lines express very low levels of 
*CYP26A1* prior to 5-aza-2’-deoxycytidine treatment. I observed a significant increase in 
CYP26A1 expression level in all three cell lines after treatment (p<0.001 for 22RV1, 
p=0.012 for DU-145 and p<0.001 for PC-3, Figure 3.5). In particular, the expression level of 
CYP26A1 was raised by 11.8 fold in PC-3.
Figure 3.4 Methylation status of \textit{CYP26A1} in three prostate cancer cell lines: A) 22RV1, B) DU-145 and C) PC-3 after both mock and 5-aza-2’-deoxycitidine treatments

5-aza-2’-deoxycitidine treatment was performed at two different time points for each cell line, each time with three technical replicates. The average \textit{CYP26A1} methylation level and error bar were calculated based on all six replicates.
Figure 3.5 CYP26A1 expression level in three prostate cancer cell lines: A) 22RV1, B) DU-145 and C) PC-3 after both mock and 5-aza-2’-deoxycitidine treatments.

5-aza-2’-deoxycitidine treatment was performed at two different time points for each cell line, each time with three technical replicates. The average CYP26A1 expression level and error bar were calculated based on all six replicates.

Basal expression of three cell lines 22RV1: DU-145: PC-3 = 1.35 : 17.36 : 1
3.3.3 Correlation of Methylation Status with Gleason Pattern

Candidate biomarkers CYP26A1 and TBX15 were further validated in the independent series of 219 prostatectomy patients using the MethyLight assay as described previously. I determined PMR threshold values that discriminated normal from cancerous tissues with the highest sensitivity and specificity using ROC curves. The cut-off values were 9.50 for CYP26A1 and 2.62 for TBX15 (Figure 3.6).

The average methylation level and HM frequency of CYP26A1 and TBX15 in prostate adenocarcinomas (stratified by GP), and their matching normals are detailed in Table 3.1. The average methylation of both genes increased significantly from normal to any tumor GP (p<0.001, Table 3.2). The proportion of HM cases was always higher in tumor than that observed in normal for CYP26A1 (p≤0.001, Table 3.2), while a substantial increase in HM frequency was observed when comparing normal with GP3, and normal with GP4&5 for TBX15 (p<0.001, Table 3.2).

3.3.4 Correlation of Methylation Status with Gleason Score

I also investigated the relationships between methylation profiles of both genes and GS. The average methylation level and HM frequency of CYP26A1 and TBX15 associated with each GS subgroup are described in Table 3.1. The average methylation level of TBX15 was significantly increased in GS7 PCa compared with GS≤6 PCa, and in GS≥8 PCa compared with GS≤6 PCa (p=0.016 and p=0.028 respectively, Table 3.2). On the contrary, no difference was observed in average methylation level for CYP26A1 among different GS groups. For both genes, the proportion of HM cases did not change substantially among various GS groups.
Figure 3.6 Optimal sensitivity and specificity determined by receiver operating characteristics (ROC) curves for the two hypermethylation gene loci (A) CYP26A1 methylation, and (B) TBX15 methylation. Maximum specificity and sensitivity achieved and cut-off value for each gene are shown.

Area under the Curve: 0.920; Sensitivity: 0.767; Specificity: 0.976

Area under the Curve: 0.894; Sensitivity: 0.786; Specificity: 0.893
Table 3.1 *CYP26A1* and *TBX15* methylation profile stratified according to Stage, Gleason Score and Gleason Pattern.

<table>
<thead>
<tr>
<th>Stage</th>
<th><em>CYP26A1</em> Methylation Status</th>
<th><em>TBX15</em> Methylation Status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average PMR</td>
<td>Percentage of HM</td>
</tr>
<tr>
<td>pT2</td>
<td>17.52</td>
<td>21.4% (30/140)</td>
</tr>
<tr>
<td>pT3a</td>
<td>24.38</td>
<td>34.5% (19/55)</td>
</tr>
<tr>
<td>pT3b/4</td>
<td>18.76</td>
<td>25.0% (6/24)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gleason Score</th>
<th><em>CYP26A1</em> Methylation Status</th>
<th><em>TBX15</em> Methylation Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤6</td>
<td>18.87</td>
<td>25.0% (25/100)</td>
</tr>
<tr>
<td>≥7</td>
<td>20.31</td>
<td>26.3% (26/99)</td>
</tr>
<tr>
<td>≥8</td>
<td>17.35</td>
<td>20.0% (4/20)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gleason Pattern</th>
<th><em>CYP26A1</em> Methylation Status</th>
<th><em>TBX15</em> Methylation Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2.37</td>
<td>0% (0/125)</td>
</tr>
<tr>
<td>2</td>
<td>19.65</td>
<td>45.8% (11/24)</td>
</tr>
<tr>
<td>3</td>
<td>19.22</td>
<td>33.5% (54/161)</td>
</tr>
<tr>
<td>4/5</td>
<td>21.18</td>
<td>36.0% (40/111)</td>
</tr>
</tbody>
</table>

HM stands for high methylation and is defined as equal or greater than the 3rd quartile PMR value of the gene of interest

Table 3.2 P-values for *CYP26A1* and *TBX15* methylation stratified by clinical characteristics

<table>
<thead>
<tr>
<th>Stage</th>
<th><em>CYP26A1</em> Methylation</th>
<th><em>TBX15</em> Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mann-Whitney U</td>
<td>Chi-square</td>
</tr>
<tr>
<td>pT2 vs. pT3a</td>
<td>0.005</td>
<td>0.057</td>
</tr>
<tr>
<td>pT2 vs. pT3b/pT4</td>
<td>0.485</td>
<td>0.696</td>
</tr>
<tr>
<td>pT3a vs. pT3b/pT4</td>
<td>0.208</td>
<td>0.402</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gleason Score</th>
<th><em>CYP26A1</em> Methylation</th>
<th><em>TBX15</em> Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤6 vs. 7</td>
<td>0.524</td>
<td>0.838</td>
</tr>
<tr>
<td>≤6 vs. ≥8</td>
<td>0.688</td>
<td>0.779*</td>
</tr>
<tr>
<td>7 vs. ≥8</td>
<td>0.224</td>
<td>0.556</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gleason Pattern</th>
<th><em>CYP26A1</em> Methylation</th>
<th><em>TBX15</em> Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal vs. 2</td>
<td>&lt;0.001</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Normal vs. 3</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Normal vs. 4/5</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2 vs. 3</td>
<td>0.705</td>
<td>0.239</td>
</tr>
<tr>
<td>2 vs. 4/5</td>
<td>0.952</td>
<td>0.369</td>
</tr>
<tr>
<td>3 vs. 4/5</td>
<td>0.679</td>
<td>0.671</td>
</tr>
</tbody>
</table>

* Fisher’s Exact Test is used instead of Chi-square Test
3.3.5 Correlation of Methylation Status with Pathological Stage

The methylation profiles of \textit{CYP26A1} and \textit{TBX15} were analyzed in relation to pathological stage. The average methylation level and HM frequency of both genes were calculated for each pathological stage group (Table 3.1). For \textit{CYP26A1}, a considerable difference in average methylation was observed between groups pT2 and pT3a (\(p=0.005\), Table 3.2), while the average methylation of \textit{TBX15} increased significantly from pT2 to pT3a, and pT2 to pT3b&pT4 (\(p=0.001\) and \(p<0.001\) respectively, Table 3.2). The proportion of HM cases of \textit{TBX15} was greater in pT3a compared with pT2, and in pT3b&pT4 compared with pT2 (\(p=0.001\) and \(p<0.001\) respectively, Table 3.2), despite a similar HM frequency of \textit{CYP26A1} across various pathological groups.

3.3.6 Predicting Biochemical Recurrence using Promoter Methylation

I next assessed the prognostic significance associated with \textit{CYP26A1} and \textit{TBX15} promoter methylation. Hypermethylation of \textit{TBX15} was significantly correlated with early biochemical recurrence in univariate log-rank test (\(p=0.003\), Figure 3.7, panel B). On the contrary, such a correlation was not observed for \textit{CYP26A1} methylation (\(p=0.600\), Figure 3.7, panel A). Following this, I stratified the patient cohort for GS and pathological stage and examined the association of methylation profiles with biochemical recurrence in each stratum. \textit{TBX15} methylation status significantly predicted early biochemical recurrence in both GS7 and GS\(\geq8\) PCa patients (\(p=0.002\) and \(p=0.040\) respectively, Figure 3.8). In multivariate analyses, GS, pathological stage and surgical margin status were shown to be significant predictors, but neither \textit{CYP26A1} nor \textit{TBX15} methylation profile could independently predict biochemical recurrence (Table 3.3-3.4).
Figure 3.7 Kaplan-Meier curves of biochemical recurrence for (A) CYP26A1 methylation status and (B) TBX15 methylation status

The blue line indicates PCa cases with low methylation, and the green line indicates PCa cases with high methylation for each gene of interest respectively. HM stands for high methylation and is defined as equal or greater than the 3rd quartile PMR value of the gene of interest, LM represents the rest of the patient cohort.
**Figure 3.8** Kaplan-Meier curves of biochemical recurrence for *TBX15* methylation status on A) GS=7 Patients and B) GS≥8 Patients

The blue line indicates PCA cases with low methylation, and the green line indicates PCA cases with high methylation for each gene of interest respectively. HM stands for high methylation and is defined as equal or greater than the 3rd quartile PMR value of the gene of interest, LM represents the rest of the patient cohort.

A

![Kaplan-Meier curve for GS=7 Patients](image)

Log rank p-value = 0.002

B

![Kaplan-Meier curve for GS≥8 Patients](image)

Log rank p-value = 0.040
Table 3.3 Multivariate Cox regression analysis of the methylation status of \textit{CYP26A1}

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>95.0% CI</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathological stage (pT2*)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT3a</td>
<td>2.746</td>
<td>1.608 - 4.690</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pT3b/pT4</td>
<td>3.310</td>
<td>1.772 - 6.183</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Surgical Margins</td>
<td>0.436</td>
<td>0.265 - 0.719</td>
<td>0.001</td>
</tr>
<tr>
<td>Age</td>
<td>1.320</td>
<td>0.827 - 2.106</td>
<td>0.244</td>
</tr>
<tr>
<td><em><em>Gleason Score (GS\leq6</em>)</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gleason Score 7</td>
<td>1.389</td>
<td>0.803 - 2.401</td>
<td>0.240</td>
</tr>
<tr>
<td>Gleason Score \geq 8</td>
<td>5.124</td>
<td>2.435 - 10.781</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CYP26A1 Methylation Status</td>
<td>0.772</td>
<td>0.442 - 1.347</td>
<td><strong>0.362</strong></td>
</tr>
</tbody>
</table>

*pT2 and GS\leq6 represent the baselines
The overall p-value associated with each clinicopathological variable is obtained by comparing the rest of the patient cohort with baseline group

Table 3.4 Multivariate Cox regression analysis of the methylation status of \textit{TBX15}

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>95.0% CI</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathological stage (pT2*)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT3a</td>
<td>2.512</td>
<td>1.456 - 4.334</td>
<td>0.001</td>
</tr>
<tr>
<td>pT3b/pT4</td>
<td>2.863</td>
<td>1.487 - 5.513</td>
<td>0.002</td>
</tr>
<tr>
<td>Surgical Margins</td>
<td>0.430</td>
<td>0.261 - 0.709</td>
<td>0.001</td>
</tr>
<tr>
<td>Age</td>
<td>1.256</td>
<td>0.790 - 1.996</td>
<td>0.335</td>
</tr>
<tr>
<td><em><em>Gleason Score (GS\leq6</em>)</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gleason Score 7</td>
<td>1.329</td>
<td>0.767 - 2.303</td>
<td>0.310</td>
</tr>
<tr>
<td>Gleason Score \geq 8</td>
<td>5.418</td>
<td>2.591 - 11.327</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TBX15 Methylation Status</td>
<td>1.311</td>
<td>0.777 - 2.210</td>
<td><strong>0.310</strong></td>
</tr>
</tbody>
</table>

*pT2 and GS\leq6 represent the baselines
The overall p-value associated with each clinicopathological variable is obtained by comparing the rest of the patient cohort with baseline group
Finally I wanted to investigate whether combining the two markers APC and TBX15 that demonstrated the most prognostic significance out of the panel of four candidate genes would better predict PSA recurrence in patients. The three groups of patients: 1) LM in both APC and TBX15, 2) HM in either APC or TBX15, and 3) HM in both APC and TBX15, were shown to be associated with significantly different rates of biochemical recurrence (Figure 3.9, p=0.005), with group 3 demonstrating the earliest recurrence rate and group 1 the latest. Nevertheless, combined methylation profiles of APC and TBX15 failed to predict biochemical recurrence independently in the presence of other clinicopathological variables (Table 3.5).

**Figure 3.9** Comparison of biochemical recurrence among 1) cases with both APC and TBX15 high methylation (HM) (indicated by beige line), 2) cases that have either APC HM, or TBX15 HM, (indicated by green line) and 3) cases with both APC and TBX15 low methylation (LM) (indicated by blue line)
Table 3.5 Multivariate analysis with combined *APC* and *TBX15* methylation profiles

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>95.0% CI</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathological stage (pT2*)</td>
<td></td>
<td></td>
<td>Overall</td>
</tr>
<tr>
<td>pT3a</td>
<td>2.231</td>
<td>1.309</td>
<td>4.114</td>
</tr>
<tr>
<td>pT3b/pT4</td>
<td>2.950</td>
<td>1.553</td>
<td>5.604</td>
</tr>
<tr>
<td>Surgical Margins</td>
<td>2.302</td>
<td>1.390</td>
<td>3.811</td>
</tr>
<tr>
<td>Age</td>
<td>1.203</td>
<td>0.754</td>
<td>1.91</td>
</tr>
<tr>
<td>Gleason Score (GS≤6*)</td>
<td></td>
<td></td>
<td>Overall</td>
</tr>
<tr>
<td>Gleason Score 7</td>
<td>1.397</td>
<td>0.804</td>
<td>2.429</td>
</tr>
<tr>
<td>Gleason Score ≥8</td>
<td>4.746</td>
<td>2.188</td>
<td>10.295</td>
</tr>
<tr>
<td>APC&amp;TBX15 Combined Methylation Status (0HM*)</td>
<td></td>
<td></td>
<td>Overall</td>
</tr>
<tr>
<td>1HM Marker</td>
<td>1.193</td>
<td>0.694</td>
<td>2.051</td>
</tr>
<tr>
<td>2HM Markers</td>
<td>1.491</td>
<td>0.761</td>
<td>2.922</td>
</tr>
</tbody>
</table>

*pT2, GS≤6 and 0HM represent the baselines*

The overall p-value associated with each clinicopathological variable is obtained by comparing the rest of the patient cohort with baseline group.
3.4 Discussions:

The differential methylation profiles of CYP26A1 and TBX15 were confirmed by EpiTYPER results at single CG dinucleotide resolution (shown in Figure 3.2-3.3). These data provided proof-of-concept that differential hybridization array could accurately capture microscopic methylation alterations.

All three cell lines treated with 5-aza-2’-deoxycytidine showed approximately 50% of demethylation of the CYP26A1 promoter which was consistent with previously reported efficiency of 5-aza-2’-deoxycytidine [192]. A correlation was shown between decreased methylation level and increased expression, suggesting that CYP26A1 was subject to methylation-mediated silencing. Previous study examining differential methylation of CpG islands in the mouse Cyp26a1 promoter across various cell lines demonstrated a key regulatory role of promoter methylation in gene transcription [193]. Methylation of Cyp26a1 promoter was correlated with minimal acetylation of histone H3, a mark known to be associated with active conformation of chromatin [193]. In line with the previous study, it appeared that promoter CpG methylation contributed to the silenced state of the CYP26A1 in PCa cell lines.

In the independent validation set, CYP26A1 showed significant diagnostic potential in differentiating normal from cancerous tissues. Aberrant promoter hypermethylation is known to be associated with gene silencing; therefore, our finding contradicted previous reports where CYP26A1 displayed oncogenic properties and elevated expression in various cancers. Even though transcriptional reactivation of CYP26A1 gene was observed for all three cell lines upon promoter demethylation, a linear correlation could not be established
between the extent of DNA demethylation and the increase in transcription level observed. Clearly, promoter methylation had a larger impact on silencing the expression level of \textit{CYP26A1} in PC-3 cell line. It seemed that other regulatory mechanisms besides promoter methylation were also involved in regulating CYP26A1 expression. Previous study has shown that a repressive state of \textit{CYP26A1} could be achieved in the absence of DNA methylation. Repressive histone modifications, namely methylation of H3K9 and hypoacetylation of histone H4 contribute to the transcriptional regulation of \textit{CYP26A1} [193, 194]. EBBP (Estrogen-responsive B Box protein, a member of the tripartite motif protein family) acts to de-repress transcription of \textit{CYP26A1} by modifying histone acetylation [195]. For the mouse counterpart \textit{Cyp26a1}, it has been observed that retinoid acid receptor$\beta$ (RAR$\beta$2) mediated its epigenetic silencing in embryocarcinoma cells in the absence of methylation [187]. An active conformation of Cyp26a1 chromatin is maintained via binding to RAR$\beta$2 and RA [187]. Furthermore, past study observed no significant changes in \textit{CYP26A1} mRNA levels in TRAMP mice as compared to the non-transgenic littermate controls [185]. Based on the above evidence, I could draw the conclusion that promoter hypermethylation is one of the many contributing factors to downregulate CYP26A1 expression in PCa cancer cells. The methylation profile of \textit{CYP26A1} is similar to that of \textit{RASSF1A}, and quantitative increase in \textit{CYP26A1} promoter hypermethylation is closely associated with PCa diagnosis but not prognosis.

\textit{CYP26A1} was chosen as a potential candidate biomarker because patients with BR exhibited pronounced promoter hypermethylation at the \textit{CYP26A1} locus compared to that of patients without BR. To my surprise, the \textit{CYP26A1} methylation profile did not correlate significantly with patient BR status in the independent validation set. I propose two reasons to explain the
observed discrepancy. First of all, the number of CpG island microarrays performed on patients with BR and patients without were very limited. With only 18 biological replicates of patients with BR, and 10 without, epigenetic changes in PCa patients could not be fully recapitulated. In addition, CpG island microarrays were performed on fresh frozen tissues from PCa patients diagnosed between the year 2001–2005; on the other hand, MethyLight data was collected from paraffin embedded tissue blocks belonging to patients diagnosed between the year 1998–2001. Since PCa is characterized by a long latency period, it is highly likely that given longer follow-up time, a certain fraction of PCa patients under the No BR category would recur. The difference in follow-up time could partially account for the discrepancy between the discovery series and the validation series.

To the best of my knowledge, prior studies have not evaluated TBX15 methylation, the gene for which I observed strong diagnostic and prognostic potentials in PCa tumours. My interest in TBX15 was due to its involvement in mesoderm layer formation in embryogenesis and the fact that loss of its expression resulted in severe skeletal malformation. The region of TBX15 gene selected on CpG island microarray was shown to be significantly differentially methylated between GS7 and GS8, and between patients with BR and patients without it. Similar findings were observed in our validation set where the quantitative methylation level of TBX15 increased with GS and PCa patients with TBX15 hypermethylation were shown to have a higher chance of biochemical recurrence. It is possible that aberrant TBX15 hypermethylation is indicative of neoplastic or pre-neoplastic cells that exist in histological normal fields surrounding the tumors. The collective methylation profiles of TBX15 and APC, which has been shown to be associated with PCa progression in the previous chapter, failed to independently predict early BR in multivariate analysis possibly due to overlapping
prediction power of these two markers. Both *TBX15* and *APC* correlated strongly with GS and stage. Therefore, additional complementary markers are needed to better predict early BR.
Chapter 4

Summary of Findings and Future Directions
4.1 Summary of Findings

In my thesis project, I have examined quantitative methylation status of two known genes: *APC* and *RASSF1A*, and two novel genes: *CYP26A1* and *TBX15*, in a series of archival prostatectomy specimens diagnosed between the years 1998 and 2001 with detailed clinicopathological follow-up data. I have shown that all four markers can distinguish normal from cancerous tissues, and therefore exhibit potential in prostate cancer diagnosis. In addition, *APC* and *TBX15* provided promising prognostic information. Quantitative changes in *APC* and *TBX15* methylation levels and HM frequencies correlated with GP, GS and pathological stage. Univariate analyses demonstrated that hypermethylation of *APC* and *TBX15* were significantly associated with early PCa recurrence. After stratification for GS and stage, *APC* was a significant predictor of biochemical recurrence for pT2 stage patients, while *TBX15* was that for GS7 and GS8 patients. Combined methylation profiles of *APC* and *TBX15* could accurately categorize patients into low, intermediate and high risk groups of biochemical recurrence. However, none of the characterized methylation markers alone or in combination could predict biochemical recurrence independently in the presence of other clinicopathological variables. My study provided proof-of-evidence that analyzing data from genomic CpG island microarrays followed by EpiTYPER and validation using MethylLight in an independent patient cohort could identify informative diagnostic and prognostic markers.

The two novel epigenetic markers *CYP26A1* and *TBX15* were identified through CpG island differential hybridization microarrays. Two recent studies published by another lab member of my lab had selected *HOXD3* using the same approach, and demonstrated a novel role of *HOXD3* hypermethylation as a potential prognostic marker of prostate cancer progression.
Collectively, these data provide proof-of-concept that using combined strategies of CpG island methylation array profiling and bioinformatics analysis, we could discover potential diagnostic and prognostic markers in PCa.

Following treatment with 5-aza-2’-deoxycytidine, the expression level of CYP26A1 mRNA increased for all three cell lines, suggesting that CYP26A1 is subject to methylation-mediated silencing. However, other regulatory mechanisms, most likely alteration of histone tails, also modify CYP26A1 expression.

One of the strengths of my project is the complete clinicopathological follow-up data available for the entire study population. I evaluated the diagnostic and prognostic utility of promoter methylation of four genes in predicting tumour progression and biochemical recurrence in PCa patients following radical prostatectomy. All four genes, APC, RASSF1A, CYP26A1 and TBX15 could accurately differentiate normal from cancerous tissues. Together they form a gene panel that could be clinically useful for the early detection and diagnosis of PCa since 1) it is very sensitive, for example, RASSF1A promoter hypermethylation was highly prevalent in tumours with higher than 95% methylation frequency; 2) it is specific, for example, APC, CYP26A1 and TBX15 methylation were rarely present in normal tissues.

An interesting aspect of my study is that GP3 and GP4 areas within GS7 tumors were analyzed separately. The general increase in the methylation levels from GP3 to GP4 for the entire cohort was also confirmed on a patient by patient basis.

I was able to establish relationships between APC and TBX15 methylation profiles with GS, stage, and biochemical recurrence, suggesting prognostic potential of these two markers. Combining their methylation profiles, I observed that the probability of early biochemical
recurrence correlated significantly with the number of genes with high methylation. My study was limited by the number of markers showing prognostic significance to significantly predict biochemical recurrence in multivariate analysis and sub-stratify histopathologically similar tumours.
4.2 Future Directions

Previous studies have reported intragenic methylation is correlated with increased transcription level. In future experiments, treatment of PCa cell lines with 5-aza-2’-deoxycytidine will help to explore the intrinsic link between TBX15 intragenic methylation and downstream expression level. I have established TBX15 hypermethylation as a progression biomarker in PCa. It would be interesting to investigate the functional contribution of TBX15 methylation using in vitro prostate cancer cell line models.

Based on my results and previous studies on CYP26A1, it seems that its transcription level in tumors is regulated by interplay between aberrant DNA methylation and modifications of histone tails. To investigate the functional significance of histone modification on CYP26A1 promoter activity and expression, PCa cell lines can be treated with Trichostatin A, a potent inhibitor of histone deacetylases.

The diagnostic and prognostic significance of the four methylation markers studied should be validated in different PCa patient cohorts to ensure the generalizability of their prediction power. Additional methylation prognostic markers could be combined together with APC and TBX15 to enhance the discretionary power in predicting PSA recurrence and ultimately patient survival. Furthermore, APC, RASSF1A, CYP26A1 and TBX15 could be exploited as predictive markers to reduce unnecessary biopsies in men with elevated serum PSA and help to identify patients who would benefit from immediate hormone therapy. It will be very interesting to see whether incorporating CpG island hypermethylation into a nomogram could improve patient management and treatment prediction.
Finally, the methylation frequencies and quantitative methylation levels of these four markers could be assessed in tissue biopsy, serum and urine samples and compared to that found in tissues. DNA methylation has been demonstrated to be readily detected in available clinical specimens obtained through non-invasive procedures, such as bodily fluids. The promoters of *APC* and *RASSF1A* have been observed to be hypermethylated in serum of breast and cervical cancer patients [196-198] and in serum and urine of renal cancer patients [199]. One study reported that the *RASSF1A* gene was hypermethylated in 24% of serum specimens from PCa patients [200]. Improved detection of PCa with DNA methylation markers in specimens obtained non-invasively, offers significant practical advantages. However, validation of promising diagnostic gene panels in large scale studies is necessary before they can be considered clinically useful.
References:


194. Gillespie, R.F. and L.J. Gudas, *Retinoid regulated association of transcriptional co-regulators and the polycomb group protein SUZ12 with the retinoic acid response*


