Exploring DNA Methylation in Tumour-Adjacent Normal Prostate Tissue and Evaluating its Role as a Biomarker for PCa Detection

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Laboratory Medicine and Pathobiology
University of Toronto

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2017

Abstract

DNA methylation alterations as a result of field cancerization may be used as diagnostic biomarkers to improve Prostate Cancer (PCa) detection. To identify diagnostic biomarkers, we evaluated DNA methylation of KRTAP27-1, APC, HOXD3, CRIP3, KLK6 and RASSF1A in tumour, tumour-adjacent normal (TAN) and benign prostate tissue. DNA methylation of APC, RASSF1A and GSTP1 was detected in TAN tissue up to 8 mm from the tumour. KRTAP27-1 was discovered to be hypermethylated in TAN tissue using Global DNA methylation profiling. Overall methylation concordance of all genes between matched radical prostatectomy (RP) tumour and RP TAN or biopsy (Bx) TAN tissue was 81% and 66%, respectively. Hypermethylation of RASSF1A, KRTAP27-1 and HOXD3 in combination were most significantly associated with PCa (Benign vs. RP TAN: $p = 5.87 \times 10^{-10}$, Benign vs. Bx TAN cases: $p = 4.44 \times 10^{-8}$). My project demonstrates the benefit of using field cancerization biomarkers for PCa diagnosis.
Acknowledgements

Firstly, I would like to thank my supervisor, Dr. Bharati Bapat for your continued support and consistent guidance. Your enthusiasm and your reassurance gave me strength to push through any challenges. You have been both a mother and a mentor towards me. Thank you helping me grow and succeed in your lab.

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Thank you to my parents and family for your love, strong faith and support. You keep me motivated to work hard and stay focused on pursuing my dreams. Thank you for believing in me and encouraging me never to give up.

Thank you to my committee members Dr. Theo van der Kwast and Dr. Susan Done for encouraging me to think beyond the boundaries of my project. Your curiosity and advice towards my research has enabled me to think more critically and explore different perspectives.

Thank you Ekaterina, Andrea, Julia, Nicole, Fang, Shivani, Madonna, Renu and Richard for your willingness to provide me with advice and assistance whenever necessary. You truly made my experience working in the Bapat Lab memorable and worthwhile. Thank you for teaching me everything that I know about working in the lab. I thoroughly enjoyed collaborating with you smart and wonderful people. A special thank you to Nicole for your guidance and getting started on this project. This work would not have been possible without your significant effort and contribution to this project.

Finally, I would like to thank Prostate Cancer Canada, Ontario Student Opportunity Trust Funds, Lunenfeld-Tanenbaum Research Institute and the Laboratory Medicine and Pathobiology Department (University of Toronto) for their financial support.
Contributions

Dr. Theo van der Kwast, Dr. Farshid Siadat and Dr. Swati Satturwar from the University Health Network (Toronto, Canada) performed histological review of tumour, TAN and/or benign prostate tissue from radical prostatectomy and/or cystoprostatectomy cases. Dr. Shuba Bellur from the University Health Network performed histological review of TAN and tumour prostate tissue from biopsy cases.

Chapter 2
Dr. Nicole White-Al Habeeb performed DNA extraction and bisulfite modification of benign and TAN prostate tissue cases analyzed for methylation using the Infinium HumanMethylation450k Bead Chip array. Dr. Pingzhao Hu (PH) at the University of Manitoba (Winnipeg, Canada) performed normalization of Infinium HumanMethylation450k Bead Chip array data and differential methylation analysis to identify differentially methylated regions. PH also provided statistical advice on how to identify candidate genes. Carmelle Fatima Cuizon performed DNA extraction, bisulfite conversion and DNA methylation analysis for the remaining data included in Chapter 2.

Chapter 3
A subset of TAN and tumour DNA cases from radical prostatectomy tissue were extracted, bisulfite converted and analyzed for the methylation of APC, RASSF1A, CRIP3 and HOXD3 by Dr. Ken Kron (former PhD student) and LiYang Liu (former MSc student). DNA extraction, bisulfite conversion and methylation analysis of TAN and tumour DNA cases from radical prostatectomy tissue were analyzed for the methylation of KLK6 by Dr. Ekaterina Olkhov-Mitsel (former PhD student). Julia Garcia performed methylation analysis on a subset of Bx TAN cases. Carmelle Fatima Cuizon performed DNA extraction, bisulfite conversion and DNA methylation analysis for the remaining data included in Chapter 3.
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# Abbreviations (in alphabetical order)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4Kscore</td>
<td>Four Kalikrein Score</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>AMACR</td>
<td>Alpha-methyl-CoA Racemase</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>BCR</td>
<td>Biochemical Recurrence</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostatic Hyperplasia</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast Cancer Type 2 Susceptibility Protein</td>
</tr>
<tr>
<td>Bx</td>
<td>Biopsy</td>
</tr>
<tr>
<td>C5orf64</td>
<td>Chromosome 5 Open Reading Frame 64</td>
</tr>
<tr>
<td>CHEK2</td>
<td>Checkpoint Kinase 2</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy Number Variations</td>
</tr>
<tr>
<td>CP</td>
<td>Cystoprostatectomy</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine base located adjacent to a guanine base</td>
</tr>
<tr>
<td>CRIP3</td>
<td>Cysteine Rich Protein 3</td>
</tr>
<tr>
<td>DMRs</td>
<td>Differentially Methylated Regions</td>
</tr>
<tr>
<td>DRE</td>
<td>Digital Rectal Examination</td>
</tr>
<tr>
<td>EPCA</td>
<td>Early Prostate Cancer Antigen</td>
</tr>
<tr>
<td>ERG</td>
<td>Transcriptional Regulator ERG</td>
</tr>
<tr>
<td>ETV1</td>
<td>ETS Variant 1</td>
</tr>
<tr>
<td>ETV4</td>
<td>ETS Variant 4</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-Fixed, Paraffin-Embedded</td>
</tr>
<tr>
<td>FOXD1</td>
<td>Forkhead Box D1</td>
</tr>
<tr>
<td>Fwer</td>
<td>Family-wise error rate</td>
</tr>
<tr>
<td>GOLPH2</td>
<td>Golgi Membrane Protein 1</td>
</tr>
<tr>
<td>GS</td>
<td>Gleason Score</td>
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<tr>
<td>GSTP1</td>
<td>Glutathione S-Transferase Pi 1</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HOXB13</td>
<td>Homeobox B13</td>
</tr>
<tr>
<td>HOXD3</td>
<td>Homeobox D3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HSD3B2</td>
<td>Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase 2</td>
</tr>
<tr>
<td>ISUP</td>
<td>International Society of Urological Pathology</td>
</tr>
<tr>
<td>KLHDC7A</td>
<td>Kelch Domain Containing 7A</td>
</tr>
<tr>
<td>KLK6</td>
<td>Kalikrein Related Peptidase 6</td>
</tr>
<tr>
<td>KRTAP3-3</td>
<td>Keratin-Associated Protein 3-3</td>
</tr>
<tr>
<td>KRTAP27-1</td>
<td>Keratin Associated Protein 27-1</td>
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<tr>
<td>LIMMA</td>
<td>Linear Models for Microarray Analysis</td>
</tr>
<tr>
<td>miRNAs</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>Mi-PS</td>
<td>Mi Prostate Score</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MYO15B</td>
<td>Myosin XVB</td>
</tr>
<tr>
<td>NCCCN</td>
<td>National Comprehensive Cancer Network</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative Predictive Value</td>
</tr>
<tr>
<td>PCa</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>PCA3</td>
<td>Prostate Cancer Antigen 3</td>
</tr>
<tr>
<td>PCPT-CRC</td>
<td>North American Prostate Cancer Prevention Trial Cancer Risk Calculator</td>
</tr>
<tr>
<td>PHI</td>
<td>Prostate Health Index</td>
</tr>
<tr>
<td>PMR</td>
<td>Percent Methylated Reference</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive Predictive Value</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific Antigen</td>
</tr>
<tr>
<td>PSADT</td>
<td>PSA Doubling Time</td>
</tr>
<tr>
<td>PSAV</td>
<td>PSA Velocity</td>
</tr>
<tr>
<td>RARB2</td>
<td>Retinoic Acid Receptor Beta</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>Ras Association Domain Family member 1</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver Operating Characteristic</td>
</tr>
<tr>
<td>RP</td>
<td>Radical Prostatectomy</td>
</tr>
<tr>
<td>SNV</td>
<td>Single Nucleotide Variants</td>
</tr>
<tr>
<td>TAN</td>
<td>Tumour-Adjacent Normal</td>
</tr>
<tr>
<td>TGFB</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TMPRSS2</td>
<td>Transmembrane Protease, Serine 2</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour, Node, Metastasis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>tPSA</td>
<td>Total PSA</td>
</tr>
<tr>
<td>TRUS</td>
<td>Transrectal Ultrasonography</td>
</tr>
<tr>
<td>UICC</td>
<td>International Union Against Cancer</td>
</tr>
<tr>
<td>VTRNA2-1</td>
<td>Vault RNA 2-1</td>
</tr>
<tr>
<td>X²</td>
<td>Chi-Squared Test</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction
1.1: The Prostate Gland

The prostate is a walnut-sized gland that mainly functions to nourish the sperm by releasing prostatic fluid containing acid phosphates, prostaglandins and proteases during ejaculation\(^1\)\(^-\)\(^3\). It surrounds the urethra below the bladder and is anterior to the rectum\(^2\). The prostate is organized into an apex, a base, and anterior and posterior surfaces (Figure 1.1). Furthermore, it is encapsulated by connective tissue that mostly consists of smooth muscle fibers and elastic connective tissue\(^2\).

The prostate is made of fibromuscular stroma and glandular tissue\(^2,4\). Fibromuscular stroma is located beneath the basal lamina that separates it from the glandular epithelial layer\(^4\). This compartment mostly consists of smooth muscle cells, fibroblasts and an extracellular matrix containing collagen fibers\(^2,4\). In contrast, the glandular tissue consists of epithelial tissue located on the opposite side of the basal lamina and secretes factors such as prostate-specific antigen (PSA) and phosphatases into the luminal space\(^5\). There are 3 zones of the prostate: the peripheral, central, and transition zone (Figure 1.1)\(^1\)\(^-\)\(^3\). The peripheral zone contains about 70% of the glandular tissue within the prostate. In contrast, the central zone consists of 25% and the transition zone consists of 5% of the glandular tissue. Prostate cancer (PCa) is most commonly found within the peripheral zone, while Benign Prostatic Hyperplasia (BPH) is often found in the transition zone\(^2,3\).
**Figure 1.1 The Prostate.** The prostate is divided into a) the central zone, b) anterior fibromuscular stroma, c) transition zone, d & f) peripheral zone and e) periurethral gland. Modified image adapted from De Marzo et al. Inflammation in prostate carcinogenesis. Nat. Rev. Cancer. 2007, 7(4):256-69.
1.2: Characteristics of Prostate Cancer

1.2.1 Epidemiology of Prostate Cancer

Prostate Cancer is the most common type of non-cutaneous cancer to affect men globally, with a higher incidence of the disease observed in developed countries\(^6\). In Canada, it was expected that 21,600 men would be diagnosed with the disease in 2016\(^7\). Although men have a 1 in 8 chance of being diagnosed with PCa in their lifetime, only 1 in 27 men with the disease are expected to die from PCa. This is due to the majority of PCa cases that are indolent or slow-growing in nature\(^7\). Globally, Canada and the US have the highest incidence of PCa. However, the incidence of PCa has been declining since 2001\(^6,7\). This may be associated with the decrease in PSA testing that had been commonly utilized since the early 1990s, but is currently not recommended for PCa screening in both countries\(^7–9\). As changes to screening techniques and novel screening technology emerge, this will likely affect the incidence of PCa worldwide.

1.2.2 Common Prostate Cancer Risk Factors

There are various established risk factors that may indicate the presence of PCa in men\(^10–12\). Age is one of the strongest risk factors for having PCa. Men with increasing age are at a higher risk of having PCa, with the incidence rising rapidly after the age of 70\(^10\). Furthermore, men over the age of 70 have the highest risk of harbouring occult disease\(^12,13\). Ethnicity is another established risk factor for PCa\(^10–12\). The incidence of PCa in patients with different ethnicities varies depending on the geographical location\(^6\). Notably, African American men have been associated with higher stage disease at diagnosis and have the highest incidence of PCa in the US\(^14\). This may be associated with socioeconomic status, limited access to healthcare and late PCa screening for this population\(^15\). Similarly, patients who have a family history of PCa are also considered to have a higher risk of developing PCa\(^10,12\). This depends on the number of relatives affected with the disease and the relationship of the patient to the affected relative. Furthermore, known genes explain approximately 35% of the familial risk for PCa\(^16\). Germline mutations in genes such as BRCA2 are often present in families with higher rates of breast and ovarian cancer\(^17\). Although it is relatively rare (1-2% of early onset PCa patients\(^10\)), men who are BRCA2 mutation carriers also have a higher likelihood of developing PCa\(^18,19\). Other gene mutations that have also been associated with an increased risk of PCa include HOXB13 and
CHEK2 genes\textsuperscript{20,21}.

1.2.3 Heterogeneity and Multifocality of Prostate Cancer

Prostate cancer is a highly heterogeneous disease in both disease presentation and clinical outcome\textsuperscript{22}. Not only can multiple tumours be found within the prostate at one time, but they can also have distinct genetic, epigenetic and morphological profiles\textsuperscript{23–27}. Some are categorized as aggressive tumours that grow quickly and can result in metastatic disease and mortality from PCa. However, the majority of PCa tumours are indolent and do not pose significant harm to patients. This type of heterogeneity between tumours makes it difficult to predict course of disease upon biopsy examination. Biopsy examination usually involves sampling of tissue from multiple locations within the prostate\textsuperscript{28,29}. Often, tumours captured upon biopsy examination can underrepresent the predicted outcome of disease due to the presence of higher grade tumours that were not captured in biopsy\textsuperscript{22}. Therefore, the multifocality of PCa limits the accuracy of pre-treatment diagnosis of PCa.

Different theories have been proposed to explain the development of multiple tumours within the prostate. One theory suggests that multiple fields or areas within the prostate gain mutations and other oncogenic properties that function synergistically to sustain the simultaneous growth of independent tumour populations\textsuperscript{24}. As the size of these lesions grow, they will often fuse to create a single heterogeneous lesion. Another theory proposes that all tumours originate from a single cell lineage and can gain the capacity to transform into distinct lesions. This could occur when tumours acquire various carcinogenic properties that can alter their molecular profile and potentially promote aggressive tumour development\textsuperscript{24}. It is possible that both theories work together to promote the multifocality of PCa.

Distinct genetic profiles have been reported in a single prostate further supporting intraprostatic heterogeneity due to multiple tumours\textsuperscript{23}. Boutros et al. (2015) observed extensive variation in the amount of copy number variations (CNV), genetic rearrangements and single nucleotide variants (SNVs) detected in separate prostate tumours from the same patient\textsuperscript{23}. Similar genetic differences have also been observed between cell populations that originate from a single tumour lesion\textsuperscript{25}. In a recent study performed by Cooper et al. (2015), distinct genetic variations
in the number and type of mutations and CNVs were observed that separated a tumour into multiple cell lineages\textsuperscript{25}.

The heterogeneity of PCa also underlies differences in disease progression that are difficult to anticipate upon diagnosis. Commonly used screening methods and evidence of prostate cancer in a biopsy cannot reliably predict the presence of aggressive PCa or course of disease. When low-grade tumours are detected upon biopsy, patients are often placed under programs called Active Surveillance to monitor the potential progression to aggressive disease\textsuperscript{30}. It has been reported that approximately one-third of patients diagnosed with low-grade PCa upon initial biopsy progress (upgrade) to intermediate-grade disease during active surveillance\textsuperscript{30}. Another significant clinical challenge is determining which PCa patients will experience biochemical recurrence (BCR) after RP (radical prostatectomy, removal of the prostate) or radiation therapy. Biochemical recurrence is defined as the rise of PSA levels after RP or therapy to indicate the presence of prostate tumour cells within the patient. Studies have shown that BCR can range from 20\% to 60\% of patients after RP or therapy\textsuperscript{31,32}. Continued investigation of PCa disease characteristics and heterogeneity will improve our ability to address the clinical challenges associated with PCa prognosis.

1.2.4 Classifying Prostate Cancer

1.2.4.1 The Gleason Grading System

The Gleason grading system was initially developed by Dr. Donald F. Gleason in 1966 to maintain consistency of tumour classification between hospital centers\textsuperscript{33,34}. It is currently one of the strongest prognostic factors for PCa\textsuperscript{35,36}. The Gleason grading system is used to classify prostate tumours based on their histological morphology and stratify patients based on their risk of having indolent vs. aggressive PCa (Figure 1.2). Prostate tumours are stratified into 5 different glandular patterns referred to as Gleason patterns\textsuperscript{35}. Gleason patterns range from 1-5, and represent morphologies of the prostate gland depending on their differentiation. Lower Gleason patterns represent well-differentiated glands, more similar to normal prostate morphology. Higher Gleason patterns represent poorly differentiated glands and indicate high risk-PCa. The Gleason grading system is synonymous with the Gleason scoring system since a Gleason score (GS) is assigned to patients to stratify them based on their risk of having low or
high grade PCa. A Gleason Score is assigned to a tumour based on the sum of two most predominant glandular patterns within the lesion. Generally, tumours assigned with a GS of 6 or less are classified as low-risk, whereas tumours that are assigned a GS of 7 and a GS of 8 or above are classified as intermediate and high risk, respectively. Multiple tumours found within the prostate may have different GS and reflect the multifocality of the disease. This makes it difficult to predict disease progression at an early stage. In recent years, the original Gleason grading system has been modified to address clinical dilemmas associated with patient risk stratification.

The most recent International Society of Urological Pathology (ISUP) Consensus Conference was held in 2014 to discuss and propose changes to the existing Gleason grading system. The intention was to make clearer guidelines for assigning GS that would more accurately reflect the disease prognosis of PCa patients in order to make proper treatment decisions. A significant modification was made to categorize the GS into 5 Grade Groups (Table 1.1). This is based on evidence that PCa patients with GS 7 tumours in which Gleason pattern 4 is predominant in the area (GS 7 (4+3) have worse prognosis compared to PCa patients with GS 7 tumours in which Gleason pattern 3 is predominant in the area (GS 7 (3+4)). Furthermore, patients with GS 8 tumours have better prognosis than GS 9-10 tumours. As a result, the newly proposed grade grouping system separates GS 7 (3+4), and GS 8 into their own categories (Table 1.1). Other guidelines proposed by this new system combines large and small cribriform architectural patterns under the Gleason pattern 4 category since both morphologies have shown a higher likelihood of poor outcomes. In addition, GS 6 tumours are also categorized under grade group 1 in an effort to clearly reflect its low risk prognosis.

Table 1.1. 2014 Gleason Grading System for Prostate Cancer Tumours. Each Grade Group corresponds to a Gleason score from the original Gleason scoring system.

<table>
<thead>
<tr>
<th>Grade Group (2014)</th>
<th>Gleason Score</th>
<th>Histological Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3+3 = 6</td>
<td>Well-formed glands; glands are not fused</td>
</tr>
<tr>
<td>2</td>
<td>3+4 = 7</td>
<td>High composition of well-formed glands with lesser composition of poorly formed or fused glands</td>
</tr>
<tr>
<td>3</td>
<td>4+3 = 7</td>
<td>High composition of poorly formed or fused glands, lesser composition of well-formed glands</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>Poorly formed or fused glands with some areas lacking glands</td>
</tr>
<tr>
<td>5</td>
<td>9-10</td>
<td>Mainly composed of areas lacking glands, with a lesser component of poorly formed or fused glands</td>
</tr>
</tbody>
</table>
1.2.4.2 Clinical and Pathological Staging

The tumour, node and metastasis (TNM) staging system was developed in 1992 by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) in order to assist with the stratification of low-, intermediate- or high risk PCa patients \(^{44}\). It categorizes prostatic tumours based on their extension into surrounding or distant areas throughout the body (Table 1.2)\(^{24,45}\). Both clinical and pathological TNM staging is performed on PCa patients to describe their predicted disease prognosis\(^ {45}\). Clinical staging is performed prior to treatment and it reflects evidence of tumour spread revealed through the use of imaging and other modalities (i.e. transrectal ultrasonography (TRUS), magnetic resonance imaging (MRI)) or digital rectal examinations (DREs)\(^ {45,46}\). In contrast, pathological staging is often performed after radical prostatectomy, where the whole organ can be assessed histologically for tumour extension within or outside of the prostate\(^ {45}\). TNM staging consists of a tumour component (T stage) that describes the spread of the tumour within the prostate or into nearby structures (Table 1.2)\(^ {44,45}\). It also consists of lymph node and distant metastasis component (N and M stage, respectively) that describes whether a tumour has metastasized into regional lymph nodes or distant organs (Table 1.2). Both clinical and pathological staging uses the same scoring system. However, a “p” is placed in front of the stage category (i.e. pT2c vs. T2c) to represent pathological staging.
Generally, TNM staging is used to help predict disease outcomes and guide treatment decisions\textsuperscript{24,45}. However, clinical staging poorly predicts pathological stage assigned after RP. This is mainly due to the multifocality of PCa. As a result, PCa is often under represented by clinical stage when compared to their final pathologic stage\textsuperscript{24,27,45}. Nomograms such as the Partin tables and Kattan nomograms combine clinical and pathological parameters (i.e. clinical stage, biopsy Gleason score, PSA) to develop a score that more accurately reflects the potential risk of non-organ-confined PCa or pathological stage at RP\textsuperscript{47,48}. These will be discussed in more detail below. Classification systems that can accurately predict the course of disease for PCa patients without the need for RP would be ideal for earlier and potentially more effective treatment.
Table 1.2. TNM staging according to the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) as of 2016.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Clinical</th>
<th>Pathological</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tx</td>
<td>Primary tumour cannot be assessed</td>
<td>-</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
<td>-</td>
</tr>
<tr>
<td>T1 (a-c)</td>
<td>Clinically inapparent tumour</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>- T1a: Tumour found in &lt; 5% of resected tissue</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- T1b: Tumour found in &gt; 5% of resected tissue</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- T1c: Tumour upon needle biopsy</td>
<td></td>
</tr>
<tr>
<td>T2 (a-c)</td>
<td>Tumour palpable and confined to prostate</td>
<td>Organ-confined tumour (No sub-classification)</td>
</tr>
<tr>
<td></td>
<td>- T2a: Tumour spread within $\leq \frac{1}{2}$ of one lobe</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- T2b: Tumour spread within $\geq \frac{1}{2}$ of one lobe, not both lobes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- T2c: Tumour spread to both lobes</td>
<td></td>
</tr>
<tr>
<td>T3 (a-b)</td>
<td>Extraprostatic tumour that does not invade adjacent structures</td>
<td>Extraprostatic Extension</td>
</tr>
<tr>
<td></td>
<td>- T3a: Extraprostatic extension</td>
<td>- pT3a: Extraprostatic extensions or invasion into bladder neck</td>
</tr>
<tr>
<td></td>
<td>- T3b: Tumour invades seminal vesicles</td>
<td>- pT3b: Seminal vesicle invasion</td>
</tr>
<tr>
<td>T4</td>
<td>Invasion of adjacent structures (except seminal vesicles): external spincter, rectum, bladder, levator muscles and/or pelvic wall</td>
<td>Invasion of rectum, levator muscle or pelvic wall</td>
</tr>
<tr>
<td>Nx</td>
<td>Regional Lymph nodes not assessed</td>
<td>Regional lymph nodes not sampled</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
<td>No positive regional lymph nodes</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in regional lymph nodes</td>
<td>Metastasis in regional lymph nodes</td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1 (a-c)</td>
<td>Distant Metastasis</td>
<td>Distant Metastasis</td>
</tr>
<tr>
<td></td>
<td>- M1a: Non-regional lymph nodes</td>
<td>- M1a: Non-regional lymph nodes</td>
</tr>
<tr>
<td></td>
<td>- M1b: Bone metastasis</td>
<td>- M1b: Bone metastasis</td>
</tr>
<tr>
<td></td>
<td>- M1c: Other sites with or without bone metastasis</td>
<td>- M1c: Other sites with or without bone metastasis</td>
</tr>
</tbody>
</table>
1.3: Prostate Cancer Screening and Diagnosis

1.3.1 Digital Rectal Examinations

Digital Rectal Examinations performed by the urologists were the primary screening tests used for PCa for many years prior to the 1980s. During this procedure, the urologist will massage the prostate through the rectum to determine if an abnormal mass can be felt to indicate the presence of a tumour. DREs are still part of common clinical practice to supplement PSA testing and contribute to clinical staging. However, there is low sensitivity and considerable variation between urologists using this method. Therefore, DREs are often used in conjunction with other tests to increase its sensitivity and accuracy of screening.

1.3.2 PSA Testing

Prostate Specific Antigen (PSA) is a glycoprotein that is released by prostatic epithelial cells to reduce the thickness of semen and enhance sperm motility. In PCa, the tissue barrier that separates blood vessels from the glandular lumen is disrupted, causing leakage of PSA into the blood. Therefore, elevated levels detected in the blood stream could indicate the presence of PCa. PSA was first discovered in 1970 by Richard J. Ablin and gained widespread clinical use in the early 1990s to screen for patients that may be at risk of PCa. The incidence of PCa rose as a result and caused increased treatment of PCa through surgery or therapy. However, there is much controversy over the continued use of PSA testing due to its limited specificity.

Patients who have serum PSA blood levels between 4.0 – 10 ng/ml are often subjected to further testing to confirm a diagnosis of PCa. However, this range of PSA blood levels is also referred to as the diagnostic grey zone since there are many non-PCa patients that have PSA levels within this range. For example, BPH and prostatitis are conditions that can enlarge the prostate organ, causing PSA levels to rise in the blood stream. These patients are often subjected to unnecessary testing, with the added risk of experiencing complications and infections. Conversely, a subset of PCa patients have low PSA levels (below 4.0 ng/ml), making it difficult to identify such patients who may benefit from further treatment if their serum PSA levels are below 4.0 ng/ml. Finally, PSA testing cannot determine which patients are likely to progress to advanced stage PCa. Therefore, patients with indolent disease may experience overtreatment in attempts to identify those with high grade PCa. PSA derivatives
and isoforms have been recommended to supplement and improve the specificity of the longstanding PSA test\textsuperscript{49,60}.

1.3.2.1 PSA Isoforms and Derivatives

Serum or total PSA (tPSA) is a combination of complexed and free PSA\textsuperscript{1,54}. Complexed PSA is the most common isoform of PSA that is found in the blood. About 70\% of PSA is bound to protease inhibitors such as alpha1-antichymotrypsin\textsuperscript{62}. However, 30\% of PSA is detected in its unbound form called free PSA. Free PSA is further broken down into three isoforms: pro-PSA, BPH-associated PSA and intact PSA\textsuperscript{49}. Measurement of free PSA has been shown to be useful in the detection of PCa. In particular, [-2]proPSA, a type of proPSA cleaved between leucine 5 and 6, is found to be elevated in serum in PCa patients and its levels may be able to aid in detection of PCa prior to initial or repeat biopsy\textsuperscript{1,54}. Furthermore, the ratio of free-to-total PSA is lower in men with PCa and improves PCa detection in men with tPSA levels between 4-10 ng/ml\textsuperscript{58,63}. Other predictive models have been developed to measure PSA isoforms in combination to improve the specificity of PCa detection.

PSA velocity (PSAV) and PSA Doubling time (PSADT) are derivatives of total PSA measurements that consider the changes in PSA levels over time\textsuperscript{1,60}. PSAV has been shown to be a strong predictor of PCa and is often used to suggest biopsy testing in patients with serum PSA levels within the diagnostic grey zone. National Comprehensive Cancer Network (NCCN) recommends its use to suggest biopsy examinations when patients have a PSAV > 0.35 ng/ml/year\textsuperscript{64}. In contrast, PSADT has been suggested as a marker to predict the need for active therapy prior to treatment. Although it has not shown promise as a diagnostic marker, it may be useful as a post treatment marker for predicting outcome and high-risk disease.

1.3.2.2 Age-adjusted PSA and PSA Density

As men age, the prostate will naturally grow in size\textsuperscript{65}. This causes an increase in the baseline levels of PSA, which can affect PSA measurements during PCa screening. Therefore, age-adjusted PSA cut offs and PSA density measurements have been proposed to account for the increased prostate volume when screening for PCa using PSA testing\textsuperscript{66,67}. Age-adjusted PSA cut offs have been shown to increase the specificity of PCa detection and reduce the number of unnecessary biopsies\textsuperscript{68}. However, many different cut offs have been proposed and further
validation of specific cut off values is necessary to confirm their clinical utility. Similarly, PSA Density is calculated by dividing the tPSA by the volume of the prostate\textsuperscript{66}. This measurement takes the size of the prostate into consideration since naturally larger prostates will produce a larger amount of PSA. This measurement was intended to distinguish between BPH and PCa as the cause of elevated PSA levels. However, its use as a predictor of PCa at biopsy is not recommended due to the difficulty in obtaining the volume measurements of the prostate\textsuperscript{69}.

1.3.2.3 Nomograms

Although conventional PSA testing alone may have limited specificity, its clinical performance may be improved when combined with other clinicopathological factors as part of a nomogram. Nomograms are predictive tools that have been developed to assess the risk of PCa or high-grade disease in patients\textsuperscript{47,48,70,71}. Clinicians will often use nomograms to help them decide whether the patient may benefit from a biopsy exam or a specific type of treatment. Usually, nomograms combine clinical and pathological information of patients (i.e. family history, age, positive or negative DRE, TRUS results, GS) to calculate a risk score that can either predict their risk of detecting PCa upon biopsy or stratify patients based on their risk of having aggressive disease after a confirmatory biopsy exam. For example, the Kattan nomogram is a commonly used predictive model that uses preoperative PSA, GS upon biopsy and clinical stage to predict the risk of biochemical recurrence\textsuperscript{48}. In addition, the North American Prostate Cancer Prevention Trial-based Cancer risk calculator (PCPT-CRC) was developed to assess the likelihood of finding PCa at biopsy and their risk of high-grade disease\textsuperscript{14}. Although nomograms may improve the predictive accuracy over any one factor alone, many types have been proposed and must be validated in multiple cohorts to assess their value for widespread clinical use.

1.3.3 Needle Biopsy Examinations for Clinical Diagnosis of PCa

1.3.3.1 Needle Biopsy Examination Schemes

Despite the emergence of novel screening tests that show promise in predicting PCa, a needle biopsy examination is still required to confirm a diagnosis of PCa. Currently, the recommended needle biopsy procedure involves the use of a TRUS-guided system to remove 10-12 cores from the prostate (Figure 1.4, A & B)\textsuperscript{72–74}. Although this is considered the gold standard for diagnosing PCa, there is currently no standardized procedure that is followed when performing
removal of cores\textsuperscript{73,74}. Hodge and colleagues introduced needle biopsy examinations for PCa detection in 1989, performing a TRUS-guided sextant biopsy procedure\textsuperscript{75}. Since then, sextant biopsies are no longer recommended due to the low cancer detection rate of high-grade PCa\textsuperscript{73}. A 12-core extended biopsy has been shown to increase the detection rate by 20-35\% compared to a sextant biopsy\textsuperscript{76-78}.

In an effort to improve PCa detection upon biopsy, a saturation biopsy procedure has been proposed, which involves the removal of 14 or more cores from the prostate\textsuperscript{77-79}. The rationale for this method is based on the thinking that more cores will lead to higher detection rates\textsuperscript{80}. However, there are conflicting results comparing the performance of saturation vs. a 12-core biopsy procedure during initial and repeat biopsy\textsuperscript{77-79,81}. Further investigation is necessary to determine the optimal conditions to use either the extended or saturation biopsy schemes for diagnosis.

1.3.3.2 Clinical Dilemmas associated with Needle Biopsy Examinations

Although TRUS-guided needle biopsies are recommended for the diagnosis for PCa, there are many limitations to using this method. When low risk PCa is detected upon biopsy, there is no way to determine if the patient may harbour aggressive PCa unless RP is performed. Many patients with initial low-risk disease detected upon biopsy experience disease up grading due to the under estimation of PCa at biopsy\textsuperscript{82}. In addition, approximately 20-30\% of negative biopsy cases are false negative\textsuperscript{83,84}. Therefore, many patients with a negative initial biopsy result will undergo a repeat biopsy examination\textsuperscript{85}. This can cause patients with indolent or no PCa to experience overtreatment, while delaying diagnosis of patients with aggressive PCa.

Patients subjected to a needle biopsy examination are also more likely to suffer from complications and infections associated with biopsy examinations\textsuperscript{86,87}. Some of these complications include hematospermia, hematuria, urinary retention and rectal bleeding. Additionally, increasing antibiotic resistance is speculated to cause hospitalization of patients due to infections\textsuperscript{87}. Post-biopsy complications can increase anxiety levels in patients, especially in those who experienced a negative biopsy result\textsuperscript{88}. These clinical issues make it imperative to find methods to optimize biopsy examinations in order to improve PCa detection and minimize the need for repeat biopsy procedures.
1.3.3.3 Transrectal vs. Transperineal Needle Biopsy Examinations

TRUS-guided biopsies are performed using a biopsy gun attached to an ultrasound probe that is directed into the prostate through the rectum (Figure 1.4 B)\textsuperscript{29}. Patients are placed under local anaesthesia to allow for the removal of 10-12 cores. Generally, this procedure can be performed within the clinic in a short period of time. Similarly, transperineal prostate biopsy procedures can also be performed, although this is a less commonly used method\textsuperscript{29}. This involves the use of a grid or guiding frame with perforations that guide the biopsy needle through the perineum to obtain prostate tissue using transrectal ultrasonography as guidance (Figure 1.4 C). With this method, approximately 20 cores or greater are removed. Although the transrectal approach is more commonly used, there are benefits to performing transperineal biopsy procedures that may improve the detection rate of PCa.

One of the main advantages of the transperineal approach is the ability to obtain prostate tissue in the anterior and apical regions of the prostate\textsuperscript{29,89}. These areas are often left unsampled during a TRUS-guided biopsy and have a higher likelihood of harbouring cancer. Furthermore, the needle is directed into the prostate parallel to the urethra. Therefore, there is less likelihood of perforating the urethra, reducing complications such as hematuria\textsuperscript{29}. Transperineal biopsy approach can also be an alternative method to minimize the occurrence of infections\textsuperscript{86,90}. Some disadvantages associated with performing transperineal biopsy examinations are due to the higher cost of equipment needed, the increased identification of indolent disease, and the complexity of the procedure\textsuperscript{29}. Since it is less commonly performed, there are fewer urologists that are familiar with this method and it may take longer to implement this method more regularly in the clinic. In addition, with the higher detection rate, more patients with indolent PCa may be identified, leading to unnecessary biopsies and further testing. Although there is evidence that the transperineal biopsy method may outperform cancer detection using TRUS-guided biopsy, both methods have advantages and disadvantages and can still miss significant cancer, leading to false negative results.

1.3.3.4 MRI-guided biopsies

MRI-guided biopsies have been shown to improve detection of PCa due to enhanced imaging of the prostate\textsuperscript{91–93}. Any suspicion of cancer observed from an MRI result is a strong predictor of disease. MRI technology can be used alone or in combination with ultrasonography and other
modalities to perform needle biopsy examinations\textsuperscript{91–93}. Multiparametric MRI modalities are also available and can improve the sensitivity and specificity of detecting PCa\textsuperscript{94,95}. However, this method is more costly and requires experienced and well-trained urologists to interpret images accurately\textsuperscript{96}. Additionally, obese patients and the presence of hip prosthetics can lower the quality of the image, making it difficult to see the organ. More investigation and trials are necessary to determine when to use this technology during clinical diagnosis.

1.4: Diagnostic Biomarkers for Prostate Cancer

1.4.1: What are Biomarkers?

A common goal of many PCa diagnostic tests is to accurately identify patients with PCa prior to or upon biopsy while minimizing the false positive and false negative results. In addition to the screening tests mentioned above, other ways to address this clinical need is with the use of biomarkers associated with PCa. Biomarkers are characterized as factors that can be measured to indicate normal or abnormal biology within a patient, or to indicate the response to therapeutic interventions\textsuperscript{97}. They can also be categorized as diagnostic, prognostic, predictive and therapeutic biomarkers to reflect their intended clinical application. Genetic, epigenetic and proteomic alterations have been investigated in PCa patients, which show strong potential as diagnostic biomarkers for early detection of disease\textsuperscript{60,\textsuperscript{98,99}}. The most clinically relevant and useful diagnostic biomarkers are ones that can be detected in biological specimens such as urine, serum and biopsy tissue, which are obtained through non- or minimally-invasive procedures.

1.4.2 Genetic Diagnostic Biomarkers

1.4.2.1 Chromosomal Rearrangements

Genetic alterations in PCa tumours are often associated with the gain or loss of expression of a gene\textsuperscript{100}. More specifically, chromosomal rearrangements often occur to dysregulate the expression of a gene. One of the most common chromosomal rearrangements observed in PCa involves a deletion on chromosome 21 to create the TMPRSS2:ERG gene fusion\textsuperscript{101–103}. TMPRSS2 is an androgen-regulated gene that encodes Transmembrane Protease, Serine 2 and is located upstream of the ERG gene, which is a part of the ETS gene family of transcription factors. As a result of this gene fusion, ERG expression is increased and causes aberrant expression of downstream target genes that promote cell motility and carcinogenesis\textsuperscript{101,103}. 
TMPRSS2:ERG gene fusion occurs in approximately 50% of PCa cases, mainly due to the heterogeneity of the disease. TMPRSS2 has also been reported to fuse with other ETS gene family members such as ETV1 and ETV4.

TMPRSS2:ERG gene fusion is speculated to occur during early PCa development with evidence that shows lower levels have been found in more aggressive disease. Therefore, TMPRSS2:ERG has been implicated as a diagnostic biomarker for early PCa diagnosis. Studies have investigated the efficacy of the TMPRSS2:ERG gene fusion to detect PCa patients using urinary sediments. Although the specificity of this biomarker is high (93%), the sensitivity is low (37%). Combining the TMPRSS2:ERG biomarker with other promising diagnostic biomarkers could improve the overall detection rate.

1.4.2.2 Long non-coding RNAs

Long non-coding RNAs are defined by their size, which range from 200 bp up to 100 kb long. They are transcribed by RNA Polymerase II and are thought to be regulatory elements that do not encode proteins. Prostate Cancer Antigen 3 (PCA3) is a long non-coding RNA molecule that is exclusively expressed in the prostate by the gene DD3. PCA3 in particular has no known function and is over expressed in PCa, making it a promising diagnostic marker. In 2012, the Food and Drug Administration (FDA) approved the use of the PCA3 as a diagnostic test called Progensa PCA3 for men over the age of 50 to aid in the decision to perform prostate biopsy exams. The PCA3 test involves the calculation of PCA3 score based on the levels of PCA3 and PSA mRNA in post-DRE urine. PCA3 score has been reported to outperform the predictive ability of tPSA to indicate PCa upon biopsy. Patients with a PCA3 score ≥ 25 are advised to undergo initial or repeat biopsy examinations to confirm the diagnosis of PCa.

1.4.3 Epigenetic Diagnostic Biomarkers

1.4.3.1 DNA Methylation Biomarkers

DNA methylation is an epigenetic modification that occurs when a methyl group is added to the 5’ carbon of a cytosine base located adjacent to a guanine base (CpG). Regions rich in CpG dinucleotides (>50%) are called CpG Islands and are often linked to the promoter region of genes. It has been reported that changes to the DNA methylation patterns are associated with
the development of various types of cancer\textsuperscript{112,113}. Generally, there is a global loss of methylation accompanied by gene-specific enrichment of methylation within the promoter regions of genes, including tumour suppressor genes and oncogenes, respectively. In PCa in particular, DNA hypermethylation has been extensively studied and hypermethylation of key genetic players, including GSTP1, APC and RASSF1A, have been reported by many groups\textsuperscript{114–117}. These genes have been investigated individually and in panels for their ability to serve as biomarkers for both PCa diagnosis and prognosis\textsuperscript{118–122}.

The identification of aberrantly methylated genes as potential PCa biomarkers has gained much interest due to the stability of DNA, which can easily be detected in urine, serum and biopsy specimens\textsuperscript{113}. GSTP1 is the most commonly hypermethylated gene in PCa tumours and is found in approximately 90\% of all cases\textsuperscript{123}, making it a promising biomarker for PCa detection. Similarly, promoter hypermethylation of APC, RASSF1A and RARB2 methylation has been extensively studied and occur frequently in PCa\textsuperscript{116,124}. Multiple hypermethylated genes have been combined to improve the diagnostic ability of any individual methylation biomarker to detect PCa\textsuperscript{121,125,126}. Furthermore, genome-wide methylation studies have also been conducted to identify novel differentially methylated genes outside of promoter regions that may also improve PCa diagnosis\textsuperscript{127–129}. Due to many methylation biomarkers that have been proposed, further validation is necessary before implementing them into clinical practice.

1.4.3.2 MicroRNA

MicroRNAs (miRNAs) are small non-coding RNA molecules that are responsible for post-translational, epigenetic regulation of gene expression\textsuperscript{130}. Typically, they are 18-25 nucleotides in length and when exported into the cytoplasm, will bind to mRNA transcripts in a complementary fashion to facilitate their repression. A single miRNA molecule can regulate the expression of over 200 transcripts\textsuperscript{130}. Therefore, the dysregulation of a single miRNA may have a significant impact on cancer development.

In PCa, miRNA expression is found to be altered in PCa and has been studied across multiple biological specimens, such as urine, tissue and serum\textsuperscript{131}. Some of the most commonly dysregulated miRNAs that have been reported in PCa include let-7a, miR-21, miR-99a, miR-141, mir-145, miR-200c, miR-221 and miR-375. Many of these miRNAs are observed to be
dysregulated across multiple specimen types. For example, it has been reported that let-7a and miR-21 is overexpressed and miR-145 is downregulated in both tissue and blood samples from PCa patients compared to healthy control patients\textsuperscript{132,133}. This characteristic makes them ideal biomarkers for early PCa detection. Furthermore, multiple miRNAs have also been combined into biomarker panels to improve the detection ability of individual miRNAs\textsuperscript{134–136}. Although the area of miRNA biology is relative new, miRNAs serve as a promising source of biological information that can be used as evidence for PCa diagnosis.

1.4.4 Proteomic Diagnostic Biomarkers

Protein expression can be altered in PCa through multiple mechanisms such as aberrant miRNA expression, chromosomal rearrangements, DNA methylation and histone modifications. Aberrantly expressed proteins have been discovered/proposed as diagnostic biomarkers for early PCa detection\textsuperscript{137–139}. As mentioned previously, PSA encodes a glycoprotein that is overexpressed in PCa\textsuperscript{1,53,54}. Although it is not PCa specific, is the most commonly used biomarker for PCa screening, despite recommendations against its use. GOLPH2 is a golgi membrane antigen protein that is also observed to be up regulated in approximately 90% of PCa patients\textsuperscript{138,140}. In addition to its high sensitivity, it can be assayed in urine, making it a promising protein biomarker for PCa detection\textsuperscript{141}. Other overexpressed proteins in PCa include Alpha-methyl-CoA racemase (AMACR) and Early Prostate Cancer Antigen (EPCA), all of which have also been proposed as potential diagnostic biomarkers for PCa detection\textsuperscript{137–139}.

1.4.5 Diagnostic Biomarker Panels

Many of the PCa biomarkers that were mentioned previously have been studied in combination to enhance the detection ability of individual biomarkers. When biomarkers are combined, their diagnostic power is added and may minimize potential false positive and false negative results. Recently, novel biomarker panels have emerged that show strong diagnostic potential and outperforms the conventional PSA screening test\textsuperscript{60}. In addition to their intended use for PCa diagnosis, some biomarker panels can also provide prognostic information prior to performing any invasive procedures. Moving forward, combining biomarkers with the strongest diagnostic potential will have the most success in clinical practice to ensure patients are given an early and accurate diagnosis.
1.4.5.1 Prostate Health Index

The Prostate Health Index (PHI) is a diagnostic test that is used to determine the probability of detecting PCa upon biopsy in patients with serum PSA levels within the diagnostic grey zone and a negative DRE\textsuperscript{60,142,143}. The PHI was approved by the FDA in 2012 and calculates a score based on the levels of tPSA, \% fPSA, and -2proPSA. In comparison to its individual PSA measurements, it has a stronger predictive ability to distinguish between benign vs. malignant prostatic conditions in men aged 50 or older, since men with PCa are more likely to have a higher PHI score\textsuperscript{142,143}.

1.4.5.2 4Kscore

Currently, there are no reliable diagnostic tests that can distinguish between low and high-risk PCa during initial screening. However, the Four-Kalikrein Score (4Kscore) has been developed to determine the risk of having aggressive PCa prior to having initial or repeat biopsy examinations\textsuperscript{144,145}. The 4Kscore is calculated based on an algorithm that combines tPSA, fPSA, intact PSA and human kallikrein 2 levels in serum to create a probability of score of 0-100\%. It will also consider clinical information of the patients, such as age, history of previous biopsy and positive or negative DRE. The 4Kscore has not been approved by the FDA, but there is evidence that it has strong potential as a pre-treatment prognostic marker to distinguish between aggressive vs. indolent PCa\textsuperscript{60}.

1.4.5.3 Mi-Prostate Score

As mentioned previously, the TMPRSS2:ERG gene fusion occurs in approximately 50\% of PCa patients\textsuperscript{101}. Although it has a high specificity, it is limited by its low sensitivity\textsuperscript{105}. However, when combined with the detection of PCA3, the sensitivity is improved from 62\% for PCA3 alone to 73\% when combined with TMPRSS2:ERG\textsuperscript{105}. A diagnostic test called the Mi-Prostate Score (Mi-PS) measures the levels of TMPRSS2:ERG and PCA3 in urine and serum PSA to create a score that predicts PCa within patients\textsuperscript{146}. Mi-PS is a commercially available test that is offered at the University of Michigan Health System and is mainly used for early PCa detection that can aid in determining the need for subsequent biopsy. A recent study reported that the test has a sensitivity of 80\% and a specificity of 90\%\textsuperscript{147}. 

1.4.6 Limitations of the Current Diagnostic Biomarkers

Many of the biomarkers mentioned above have been proposed to improve the detection of PCa\textsuperscript{98}. Ideally, biomarkers that are stable and can easily be detected in biofluids (i.e. urine, serum, whole blood) are favourable since they can be obtained from patients without performing invasive procedures. However, many different biomarkers have been proposed and require extensive validation in large multi-national cohorts before being implemented for widespread clinical use. Therefore, the current gold standard for PCa diagnosis is a histopathological examination of a needle biopsy, which is also limited in its ability to detect PCa due to sampling bias\textsuperscript{72–74}. If PCa is not detected on biopsy, there are currently no reliable diagnostic tests that can confirm whether patients who received an initial negative biopsy result are positive for the PCa. Despite the emergence of many promising diagnostic biomarkers for PCa, markers that can specifically identify patients with PCa upon negative biopsy are warranted.

1.5: Field Cancerization

1.5.1 What is Field Cancerization?

It has been proposed that the prostatic tissue microenvironment upon which a tumour lesion grows may develop molecular, genetic and epigenetic aberrations before any histological evidence of cancer is observed (Figure 1.5\textsuperscript{148–151}). This process is called field cancerization. Various molecular changes and environmental carcinogens can interact in the prostate to create a field of disturbed tissue and stroma\textsuperscript{149–151}. With the right combination of events and cellular states, the microenvironment can be sufficiently primed to cause the accumulation of other mutations and alterations within this area\textsuperscript{24,148,149}. This can activate oncogenic pathways and ultimately promote PCa development. These changes often precede any histological changes that indicate the presence of disease. In particular, genetic and epigenetic changes have been observed in tumour-adjacent normal (TAN) prostate tissue as a result of field cancerization\textsuperscript{150–152}. These have been proposed as potential diagnostic biomarkers, especially in patients with an initial negative biopsy result\textsuperscript{120,153}. Ideally, genetic and epigenetic biomarkers identified in negative biopsy tissue can improve PCa screening by allowing for early detection and minimizing the number of repeat biopsy examinations in patients who are cancer-free.
1.5.2 Epithelial & Stromal Changes in Field Cancerization

The prostate is composed of two main compartments – epithelial tissue and the stroma\textsuperscript{154,4}. In the context of PCa, the transformation of both compartments may contribute to the development of tumour lesions. Prostatic epithelial cells will often undergo genetic and epigenetic transformations that enhance the activity of oncogenic pathways. Stromal cells can also acquire molecular alterations that promote tumourigenesis. In tumour-associated stroma, there is an increase in the proportion of fibroblasts and myofibroblasts and a decrease of smooth muscle cells observed\textsuperscript{154,4}. This is commonly described as Reactive Stroma, since it mimics the molecular composition of stromal compartments in wounds\textsuperscript{148,154-5}. Normally, stromal and epithelial cells interact to regulate normal prostate development and differentiation. Therefore, it is possible that alterations in both compartments can modify their interactions and contributes to the development of field cancerization (Figure 1.6).

Interactions between prostate stroma and epithelial tissue occur via receptor signalling. For instance, stromal cells will release growth factors (i.e. fibroblast growth factor, epidermal growth factor) that are bound by their corresponding receptor located on normal prostatic epithelial cells to promote normal cell growth\textsuperscript{5,154}. However, the changes that occur in each compartment can alter their interactions to promote field cancerization and tumour development. For example, stromal cells release TGFB to inhibit the growth of epithelial cells\textsuperscript{5}. However, if
epithelial cells often lose their ability to express TGFB receptors, they become insensitive to TGFB. This increases the propensity of cells to grow. With increased levels of TGFB in stromal cells, this can induce the differentiation of fibroblasts into myofibroblasts, leading to synthesis of Reactive Stroma that can improve cell motility.

As a result, this area of tissue has gained the characteristics that are optimal for tumour spread. In combination with other molecular aberrations, field cancerization will continue to develop in this area and further promote tumourigenesis (Figure 1.6).

**Figure 1.6. Epithelial and stromal cell interactions in Field Cancerization.** When epithelial or stromal cells are exposed to stimuli that alter their genetic, epigenetic or molecular profile, their interactions can cause tumour development. This describes Field Cancerization. Black arrows represent interactions that promote carcinogenesis.
1.5.3 DNA Methylation in Field Cancerization

Global loss and gene-specific gain in DNA methylation is a key epigenetic feature of cancer\textsuperscript{112}. In PCa, DNA hypermethylation has been extensively studied and often occurs in promoter CpG islands, as mentioned previously\textsuperscript{114}. Interestingly, many hypermethylated genes observed in PCa tumours have also been observed in the TAN tissue\textsuperscript{127,155–157}. This supports the idea of field cancerization where cells within a given tissue microenvironment acquire alterations that predispose the development of tumours. Therefore, DNA methylation is considered an early event in carcinogenesis and represents a potential biomarker source that may indicate the presence of PCa at an early stage.

Differentially methylated genes can easily be interrogated in many specimens, such as plasma, serum, urine, and tissue\textsuperscript{158}. Furthermore, the hypermethylation of tumour suppressor genes RASSF1A, APC and GSTP1 that are frequently found in tumours have also been observed in adjacent normal tissue\textsuperscript{151,155,157}. These characteristics make DNA methylation biomarkers in TAN tissue ideal for PCa diagnosis, especially if they can be detected in negative biopsy tissue. It is likely that negative biopsy tissues are removed from TAN prostate tissue. Therefore, it is possible that differentially methylated genes associated with PCa can indicate the presence of disease if they can be detected in negative biopsy tissue. This will prevent the need to repeat biopsy examinations and identify patients who may need more aggressive treatment in a timely manner.

1.5.4 Confirm MDx

Confirm MDx is a commercially available assay that uses methylation-specific qPCR technology to detect DNA promoter hypermethylation of GSTP1, APC and RASSF1A in negative biopsy tissue\textsuperscript{159–162}. This test uses the concept of field cancerization to identify whether a patient must undergo a repeat biopsy examination (Figure 1.7). If a patient has methylation of any one of the three genes in any of their negative biopsy cores, they are advised to undergo repeat biopsy examinations. Due to its high negative predictive value (NPV) (89%), there is a high likelihood that the ConfirmMDx assay can ensure all patients with no methylation of GSTP1, APC and RASSF1A are cancer free to avoid unnecessary repeat biopsy procedures.
However, the positive predictive value (PPV), sensitivity and specificity of this test is 29%, 68% and 64%, respectively\textsuperscript{159,160}. Therefore, approximately 30% of patients with a positive test result may actually be negative for PCa.

Although ConfirmMDx may lower the number of unnecessary biopsy procedures, there are a significant proportion of cancer-free patients that may have a positive test result and undergo unnecessary biopsy procedures. Based on the performance of this test, GSTP1, APC and RASSF1A may not be exclusively PCa-associated markers and can indicate the presence of other conditions within the prostate. The addition of more DNA methylation biomarkers to this panel may improve the performance of this test and prevent false positive results that lead to overtreatment.

**Figure 1.7. ConfirmMDx Biopsy Examination Schematic.** A) Map of the prostate that reveals areas where biopsy cores are often taken, missing the tumour focus. B) Map of the prostate reveals biopsy cores that overlap areas around the tumour that are affected by field cancerization. Modified image adapted from mdxhealth.com.
1.6 Hypothesis, Major Goal and Specific Aims

1.6.1: Hypothesis

I hypothesize that differentially methylated genes identified in TAN tissue may be used as field cancerization biomarkers to improve PCa detection and/or prognostication.

1.6.2: Major Goal

My major goal is to identify promising DNA methylation biomarkers that can be used for PCa diagnosis in negative biopsy tissues by evaluating the DNA methylation pattern of known and novel genes in TAN prostate tissues. Newly identified biomarkers may be used in combination with existing biomarkers to improve PCa and minimize the overtreatment of patients. This will reveal the importance of field cancerization for clinical assessment of PCa.

1.6.3: Specific Aims

1. To characterize the DNA methylation profile in TAN prostate tissue in order to identify and validate novel differentially methylated genes and determine the extent of field cancerization surrounding prostate tumours.

2. To evaluate the methylation of a selected panel of known and novel genes in matched tumour, TAN and benign prostate tissue to determine their ability to detect PCa patients.
Chapter 2: Discovery and Validation of Novel Differentially Methylated Genes in Tumour-Adjacent Normal Prostate Tissue
2.1 Introduction

When patients who are suspected to have PCa undergo a needle biopsy examination, approximately 25-30% of patients may receive a false negative result\textsuperscript{83,84}. Therefore, patients with an initial negative biopsy result will often undergo a repeat biopsy examination. This will cause many patients who do not have PCa to be subjected to unnecessary procedures. Biomarkers that can be detected in negative biopsy tissue to indicate the presence of PCa are necessary to minimize false negative results and prevent the overtreatment of patients who do not have PCa.

As mentioned previously, field cancerization is a phenomenon that causes genetic, epigenetic and proteomic changes to occur in histologically normal appearing tissue surrounding a tumour\textsuperscript{150,151}. A common epigenetic alteration that occurs in PCa is DNA methylation, which has been reported in TAN tissue as a result of field cancerization\textsuperscript{121,126,127,155,157,163}. Negative biopsy cores from PCa patients are likely to be removed from TAN areas and harbour DNA methylation changes indicative of carcinogenesis. Therefore, differentially methylated genes in TAN tissue may be used as biomarkers to detect PCa in negative biopsy tissue. In this chapter, I characterized the DNA methylation profile in TAN tissue to identify differentially methylated genes that can potentially be used as biomarkers for PCa detection in negative biopsy tissue.

2.1.1 Global DNA Methylation Profiling to Identify Diagnostic Biomarkers

The commercially available ConfirmMDx assay partially address the dilemma associated with identifying PCa patients who have received an initial negative biopsy result\textsuperscript{159,160}. With a high NPV, this assay can make strong assumptions that patients without the methylation of APC, GSTP1 or RASSF1A in their negative biopsy cores are negative for PCa. However, approximately 30% of patients with a positive assay result are also negative for PCa and may still undergo unnecessary biopsy procedures. Therefore, more robust biomarkers that have a higher sensitivity and specificity are necessary to ensure only PCa patients undergo further treatment.
Ideal biomarkers for PCa detection in negative biopsy tissue are those that have a higher methylation signal in TAN compared to benign prostate tissue from non-PCa patients. As mentioned previously, DNA hypermethylation in PCa is often found in promoter regions that are rich in CpG sites\textsuperscript{163}. However, it is possible that other genomic regions outside of promoter regions may also exhibit robust differential methylation as a result of field cancerization. Therefore, we performed global DNA methylation profiling using the Illumina HumanMethylation450 BeadChip array\textsuperscript{164}. This array can detect the methylation pattern of over 480,000 CpG sites using a small amount of DNA per sample. CpGs interrogated by this array are associated with approximately 96% of RefSeq genes and 99% of all CpG islands within the genome. The benefit of using this array is that it investigates the methylation of CpG sites located outside of promoter regions, which are less studied in the context of PCa\textsuperscript{164}. By using this platform to analyze the methylation patterns in TAN vs. benign prostate tissue, we have a greater likelihood of discovering robust differentially methylated regions or CpG sites across the genome that may be used as diagnostic biomarkers to detect PCa patients using negative biopsy tissue.

2.1.2 Characterizing the Extent of Field Cancerization

It is likely that patients with PCa will have negative biopsy tissue taken from TAN areas affected by field cancerization\textsuperscript{121,126,127}. However, the ability to detect DNA methylation alterations associated with PCa in negative biopsy tissue depends on how far field cancerization occurs around the tumour itself and where the biopsy core was removed in relation to this area. Therefore, characterizing the extent of field cancerization in TAN prostate tissue is necessary to determine the target area surrounding a tumour upon which DNA methylation alterations can be detected. Conversely, it will also assist in estimation of the distance where a tumour focus lies in relation to where the biopsy core was removed. To do this, we evaluated the methylation patterns of APC, GSTP1 and RASSF1A at increasing distances away from the tumour. APC, GSTP1 and RASSF1A promoter hypermethylation has been identified in both tumour and TAN prostate tissue and used as a part of the ConfirmMDx panel of biomarkers\textsuperscript{161}. Therefore, we analyzed the methylation of these genes in TAN tissues at varying distances from a tumour focus to indicate the extent of field cancerization.
2.2 Materials and Methods

2.2.1 Patient Cohort

Prostate tissue was obtained from twenty-four patients to perform global DNA methylation profiling. TAN and benign cystoprostatectomy (CP) tissues from radical prostatectomy (RP) or benign prostate tissues from CP cases were microdissected for DNA methylation analysis. Eight patients underwent CP and had no histological evidence of PCa within the prostate. The remaining 16 patients underwent a RP due to histological evidence of PCa upon biopsy. Among these, eight patients had low-grade cancer (≤ GS 6 tumours), and 8 patients had high-grade cancer: 4 with GS 8 tumours and 4 with GS 9 tumours. To validate the methylation patterns of our candidate genes in benign CP and TAN prostate tissue, an independent cohort of 69 patients with benign CP tissue (n = 11) or low- (n = 26) or intermediate- (n = 32) grade prostate tumours were recruited. This independent cohort represents Cohort 2. An additional cohort of 18 PCa patients were recruited to determine the distance that altered DNA methylation patterns can be detected in TAN tissue surrounding a tumour. All patients were recruited from the University Health Network, Toronto, Canada following Research Ethics Board review and approval.

2.2.2 DNA Extraction and Bisulfite Conversion

Radical prostatectomy and cystoprostatectomy tissue sections from each patient were obtained from formalin-fixed, paraffin-embedded (FFPE) blocks. One representative block per case was obtained and cut into 7-10 μm serial sections with up to 5 sections per case. The first slide was stained with hematoxylin and eosin (H&E) for each case and TAN areas were marked by Dr. T. van der Kwast. Similarly, H&E slides were prepared for each cystoprostatectomy case and benign CP areas were marked by Dr. F. Siadat. H&E slides were superimposed over the unstained serial sections of tissue to mark the TAN or benign CP areas for microdissection. Extraction was performed using either the QIAmp® DNA Mini Kit (Qiagen, Mississauga, On, Canada) using a modified protocol165 or the ReliaPrep™ FFPE gDNA Miniprep System (Promega, Madison, WI, USA). DNA was quantified using Nanodrop 8000 Spectrophotometer (Thermo Scientific, Wilmington, USA). 100 ng or greater of total DNA was bisulfite-converted.
using EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) using the protocol provided.

2.2.3 Global DNA Methylation Profiling Using Illumina Infinium HumanMethylation450 Bead Chip Array

500-700 ng of bisulfite-converted DNA from each case was sent to The Center for Applied Genomics at The Hospital for Sick Children Research Institute by Dr. N. White-Al Habeeb (past Post Doctoral Fellow in the lab) to perform genome-wide methylation profiling using the Illumina Infinium HumanMethylation450 BeadChip Array. This platform allows for the simultaneous methylation analysis of over 480,500 CpG using minimal DNA input\textsuperscript{164}. A single BeadChip can accommodate up to 12 samples for methylation quantification. The quantification of each CpG site involves the use of two chemical assays: Infinium I and II\textsuperscript{164}. The Infinium I assay involves the binding of either an unmethylated bead-type or methylated bead-type probe that will discriminate between methylated or unmethylated CpG sites. The infinium II assay uses beads that will bind to a region of interest and allow for single-base extension using biotin-labelled dNTPs. dNTPs will then emit a signal to indicate whether a specific CpG site is methylated. Fluorescent channels will detect the methylation status of each CpG site interrogated and quantify the methylation signal based on its intensity. Beta (\(\beta\)) values are used to quantify the level of methylation within a CpG site and range from 0-1\textsuperscript{166}. \(\beta\) values closer to 0 represent low or no methylation, while \(\beta\) values closer to 1 represent high methylation.

The Illumina GenomeStudio software was used to perform signal normalization and background subtraction to process the data for further analysis\textsuperscript{164}. Functional normalization and quantile normalization were both applied to the microarray data separately to remove unwanted variation, control for batch effects, and remove probes that contribute to background noise\textsuperscript{164,167,168}. Beta (\(\beta\)) values were then assigned to each probe that corresponds to a distinct CpG site to quantify their methylation status.
2.2.4 Identifying Candidate Gene-Associated Probes

Differential methylation analysis was performed on the microarray data to identify potential differentially methylated CpG sites and regions for further validation. Out of the 24 samples analyzed using the Illumina HumanMethylation450 BeadChip array, only 19 samples could be used for differential methylation analysis due to poor DNA quality. Linear Models for Microarray Analysis (LIMMA) and methyAnalysis were applied separately to identify significant differentially methylated CpG sites between groups after quantile normalization was performed\textsuperscript{169,170}. In both analyses, β values were log transformed into M values to quantify the methylation value of each CpG site \( M = \log_2 \left( \frac{\beta}{1-\beta} \right) \). Bayesian t-tests were used to compare the methylation of all interrogated CpG sites between TAN and benign CP samples, and TAN samples stratified by low and high-risk cases. Differentially methylated CpG sites with an unadjusted (unadj.) \( p \leq 0.05 \) were considered significant. Similarly, Bump hunting (performed by Dr. P. Hu, University of Manitoba) was also applied to identify significant differentially methylated regions (DMRs) after functional normalization was performed\textsuperscript{171}. Regions of the genome that include highly correlated, CpG methylation levels were compared between groups. DMRs with a family-wise error rate (fwer) \( \leq 0.25 \) were considered significant.

2.2.5 Quantitative Methylation-Specific PCR

The quantitative, methylation-specific PCR technique called Methylight was used to validate the methylation patterns of our candidate biomarkers and analyze the methylation patterns of known biomarkers to measure the extent of field cancerization (Figure 2.1)\textsuperscript{172–174}. Primers and probes were designed for our CpG sites or regions of interest associated with our candidate genes KRTAP3-3, KLHDC7A, KRTAP27-1 and VTRNA2-1 (Table 2.1). Each tube containing lyophilised primers and probes were suspended in 1x TE buffer (pH 8.0) to a final concentration of 100 mM. Oligomix was created using 3 ul probe solution, 9 ul forward primer solution, 9 ul reverse primer solution and 79 ul H\textsubscript{2}O for 100 reactions.

Methylight was performed on the QuantStudio 6 Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA, USA). Standard curves were created using positive control super-methylated DNA at a 1:4 dilution factor. ALU-C4 is used as an input control to normalize the
amount of DNA added. Percent Methylated Reference (PMR) values were calculated to quantify the methylation of our genes within each DNA sample. PMR values were calculated as follows: 

\[
\text{PMR} = \left( \frac{\text{Gene of interest/ALU fluorescence quantity ratio for sample DNA}}{\text{Gene of interest/ALU fluorescence quantity ratio for super-methylated DNA}} \right) \times 100\%.
\]

Each DNA sample was analyzed in duplicate.

**Figure 2.1 Methylight: Quantitative, methylation-specific qPCR assay.** Double-stranded DNA is represented by the horizontal red lines. DNA located within the orange box represents unmethylated DNA. DNA located within the green box represents methylated DNA. Methylated DNA is bound by primers and probes to allow for the amplification of our gene of interest. Taq Polymerase cleaves the fluorescent probe that will emit a fluorescent signal. DNA methylation level of our gene is quantified based on the amount of amplification detected by the amount of fluorescence emitted. Image adapted from Dr. E. Olkhov-Mitsel.
Table 2.1 Primer and Probe sequences used for methylation analysis of genes using Methylight qPCR technology.

<table>
<thead>
<tr>
<th>Primer/Probe Sequences</th>
<th>Forward</th>
<th>Probe</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLHDC7A</td>
<td>ATT TGT TTT AAT GAG AGA TTT TGT ATT ACG</td>
<td>CAC AAC AAA ACC TTA AAC CCG CAC TCT CAA</td>
<td>AAT TAA ACA ACT AAC CCA AAA AAA ACG</td>
</tr>
<tr>
<td>KRTAP27-1</td>
<td>TGA AGA ATT TTT TGA TTG GTA CG</td>
<td>CTA CTT ACT CCA ATT CCA AAC CCT ACG AAA A</td>
<td>TAT AAC ATA ACT ACT TCC CCG</td>
</tr>
<tr>
<td>HSD3B2</td>
<td>AAT CTA AAA AAA CCA AAA AAA CGC</td>
<td>TAT CGG GGA AGA GTT ATT TTT TAT CGG TTT T</td>
<td>TTT TGA GTT AGT TTG TTT AAA GTC G</td>
</tr>
<tr>
<td>C5orf64</td>
<td>TTT TAG ATT GGT TAA TAG GAG ATC G</td>
<td>GGT CGT GGT CGT TGT ATT TTG TTC GAG A</td>
<td>ACC CCT ATT CCA AAA ACC G</td>
</tr>
<tr>
<td>KRTAP3-3</td>
<td>GAAAGATTGTTGTTTATGATCTTTGACG</td>
<td>AAC CAC AAA CCC ACC TTA TAC TCC CTC TT</td>
<td>AAC CAA ACT AAA CGA AAA TAA TTC G</td>
</tr>
<tr>
<td>VTRNA2-1</td>
<td>TGACGATGTGGAGAGGGAC</td>
<td>AGAGGTTTGCGTTATGCGGTTTCGTTCGTTT</td>
<td>CAC GTT AAC AAA ACG CCT AAC G</td>
</tr>
<tr>
<td>GSTP1</td>
<td>GTC GGC GTC GTG ATT TAG TAT TG</td>
<td>AAA CCT CGC GAC CTC CGA ACC TTA TAA AA</td>
<td>AAA CTA CGA CGA CGA AAC TCC AA</td>
</tr>
<tr>
<td>APC</td>
<td>GAA CCA AAA CGC TCC CCA T</td>
<td>CCC GTC GAA AAC CCG CCG ATT A</td>
<td>TTA TAT GTC GGT TAC GTG CGT TTA TAT</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>ATT GAG TTG CGG GAG TTG GT</td>
<td>CCC TTC CCA ACG CGC CCA</td>
<td>ACA CGC TCC AAC CGA ATA CG</td>
</tr>
<tr>
<td>HOXD3</td>
<td>TTC GGA ATA GTT AGA GAT TAA AGT GC</td>
<td>ACA AAA CGT TCC CGA CGC TTC TAA AA</td>
<td>CCA AAA TAA TAA AAC AAA ACG AAA CGA C</td>
</tr>
<tr>
<td>KLK6</td>
<td>AGG AAG TTA TTG ATG TAATCG TTT TTT CG</td>
<td>ACT CGG ACC TCA ACC TCT CTT CCG ACG AAC A</td>
<td>AAA ACA ATC GAACTT TAT CCG CC</td>
</tr>
</tbody>
</table>
2.2.6 Statistical Analysis

Final PMR values for each gene of interest were determined by calculating the mean PMR of two replicates. If two replicates had PMR values that were more than 10% apart from each other, a 3rd replicate was run and the mean PMR of all three replicates was calculated. Mann-Whitney U test was performed to compare methylation patterns between two groups. Kruskal-Wallis Test was performed to compare methylation patterns between 3 or more groups. Paired analysis was performed using Wilcoxon Ranked Sum test for all matched samples. Statistical analysis was performed using SPSS software (v. 21, Chicago, Illinois, USA).

2.3 Results

2.3.1 Determining the Extent of Field Cancerization affecting DNA methylation in TAN tissues

Negative biopsy cores removed from the prostate are likely to come from TAN tissues surrounding a prostate tumour. However, it is unclear how far DNA methylation changes in TAN tissues can be detected from the tumour focus itself. Therefore, we analyzed the methylation patterns of known genes at varying distances away from the tumour using Methylight technology. APC, GSTP1 and RASSF1A methylation has previously been reported in TAN prostate tissue and is currently used in combination to create The ConfirmMDx biomarker panel\textsuperscript{155,157,159}. Therefore, we selected these genes to analyze the extent of field cancerization due to the higher likelihood of detecting their methylation in TAN prostate tissue. APC, GSTP1 and RASSF1A methylation was analyzed in 24 RP tumour and matched TAN tissue at 0-4 mm and 4-8mm away from the tumour. The methylation of all three genes was higher in tumour compared to TAN prostate tissue up to 8 mm away (Figure 2.2, p < 0.05). Interestingly, a significant decrease in the methylation was observed for GSTP1 in TAN tissue 4-8 mm away from the tumour (Figure 2.2, p = 0.008). In contrast, the methylation levels for APC and RASSF1A were consistent in TAN tissue up to 8 mm away from the tumour (Figure 2.2, p > 0.05).
2.3.2 Characterization of the Global DNA methylation profile in TAN Prostate Tissue

To explore the genome-wide methylation alterations as a result of field cancerization, we performed global DNA methylation profiling on TAN and benign CP prostate tissue using Illumina HumanMethylation450 BeadChip Array. Due to low sample quality, only 19 out of 24 samples were included in the analysis. methyAnalysis identified 1,152 differentially methylated CpG sites (unadj. p < 0.05, Fold Change (FC) > 2) (Figure 2.3 A). Similarly, LIMMA analysis identified 3,472 differentially methylated CpG sites (unadj. p < 0.05, FC > 2) (Figure 2.3 B). Unsupervised hierarchical clustering revealed a distinct methylation pattern between TAN and benign CP cases for these identified differentially methylated CpG sites. To narrow down our search for candidate DNA methylation biomarkers, we applied more stringent criteria (unadj. p < 0.01, FC > 4 and Δβ ≥ 0.2) and selected differentially methylated CpG sites identified by both LIMMA and methyAnalysis. In total, 934 differentially methylated CpGs were selected. Unsupervised hierarchical clustering also revealed a distinction between the methylation pattern of all TAN vs. benign CP cases (Figure 2.3 C).
2.3.3 Identification of Candidate DNA Methylation Biomarkers for PCa Detection

Out of the 934 differentially methylated CpG sites identified by LIMMA and methyAnalysis, 5 CpG sites were selected based on their robust differential methylation pattern observed ($p \leq 0.01$, FC $\geq 4$ and $\Delta\beta \geq 0.2$). Each CpG site is associated with a specific gene: KRTAP27-1, KRTAP3-3, HSD3B2, KLHDC7A and C5orf64 (Figure 2.4). LIMMA and methyAnalysis can only identify differentially methylated CpG sites. To identify DMRs, Bump hunting analysis was applied to our microarray data. After comparing methylation between benign CP vs. TAN cases, we decided to focus on the comparison between benign CP vs. all TAN cases or benign CP vs. TAN GS6 cases. Ideally, this would allow us to identify candidate biomarkers that may be associated with early PCa development. As a result, 3 DMRs were identified (Figure 2.4, fwer $< 0.25$). Two DMRs were identified in the comparison between benign CP vs. all TAN cases (MYO15B, fwer = 0.224 and uncharacterized DMR 100 kb away from FOXD1, fwer = 0.192, Figure 2.4) and 1 DMR was identified between benign CP vs. TAN GS 6 cases (VTRNA2-1, fwer = 0.022, Figure 2.4).
Figure 2.3. Global DNA Methylation Profiling of TAN vs. Benign CP Prostate Tissue. A) Top differentially methylated genes from methyAnalysis (un-adjusted $p<0.01$, FC > 2). B) Top differentially methylated genes from LIMMA Analysis (un-adjusted $p<0.01$, FC > 2). C) Top differentially methylated genes from both LIMMA and methyAnalysis (unadj. $p < 0.01$, FC > 4 and $\Delta \beta > 0.2$) (Benign CP: $n = 6$, TAN GS6: $n = 7$, TAN GS89: $n = 6$).
Figure 2.4. Schematic diagrams that represent the location of candidate CpG sites or DMR in relation to associated gene. Black boxes represents amplified region for Methylight analysis. Green boxes represent nearby CpG islands.

**KRTAP27-1**
Location of Gene: chr21:31,709,331-31,710,012
Candidate CpG site: cg05809586 (chr17: 31,709,691)
Number of Exons: 1
Amplified region for Methylight: chr17: 31,709,630-31,709,751

**VTRNA2-1**
Location of Gene: chr5:134,416,187-134,416,286
DMR includes the following CpG sites: cg18678645, cg06536614, cg26328633, cg25340688, cg2686946, cg00124993, cg08745956, cg16615357 (chr5: 135,416,331-135,416,613)
Number of Exons: 1
Amplified region for Methylight: chr5: 35,416,328-135,416,426

**KLHDC7A**
Location of Gene: chr1: 18,807,424-18,812,480
Candidate CpG Site: cg23688827 (chr1: 18,767,776)
Number of Exons: 1
Amplified region for Methylight: chr1: 18,767,116-18,767,837

**HSD3B2**
Location of Gene: chr1: 119,957,270-119,965,662
Candidate CpG Site: cg17951978 (chr1: 119,960,008)
Number of Exons: 3
Amplified region for Methylight: chr1: 119959947-119960067

**C5orf64**
Location of Gene: chr5: 60,920,228-60,923,894
Candidate CpG Site: cg13634242 (chr5: 60,921,891)
Number of Exons: 3
Amplified region for Methylight: chr5: 60,921,831-60,921,952

**KRTAP3-3**
Location of Gene: chr17: 39,149,682-39,150,385
Candidate CpG Site: cg13183651 (chr17: 39,151,857)
Number of Exons: 1
Amplified region for Methylight: chr17: 39,151,797-39,151,918

**Uncharacterized DMR**
Nearest gene: FOXD1 (chr5: 72,742,085-72,744,352)
DMR includes the following CpG Sites: cg11176481, cg01558931 (chr5: 72,676,039-72,676,121)
2.3.4 Technical Validation of Candidate Biomarkers using MethyLight Technology

Technical validation of our genes of interest was performed on TAN (n = 11) and benign CP (n = 6) samples from the array using MethyLight technology. Primers and probes were designed to interrogate the methylation patterns of KRTAP27-1, KRTAP3-3, HSD3B2, KLHDC7A, C5orf64 and VTRNA2-1 within the CpG site or region of interest. Unfortunately, the primer/probe sequences for C5orf64 and HSD3B2 were unable to amplify our region of interest and could not be used for qPCR methylation analysis. This may be due to non-specific binding or inability to bind due to unmethylated CpG sites within the primer or probe sequence. Comparative analysis was performed between benign CP and all TAN cases for KRTAP27-1, KRTAP3-3, VTRNA2-1 and KLHDC7A (Figure 2.5 A&B). Methylation of KRTAP27-1 and KLHDC7A was significantly elevated in TAN cases (Figure 2.5 A, p = 0.028 for KRTAP27-1, p = 0.02 for KLHDC7A). After stratifying TAN cases by low and high-risk disease, KRTAP27-1 and KLHDC7A methylation in TAN GS 6 (n=6) was significantly increased compared to benign CP cases (Figure 2.5 B). In contrast, methylation of KRTAP3-3 and VTRNA2-1 was similar between benign CP and TAN cases (Figure 2.5 A&B, p > 0.05).
Figure 2.5. Validation of our genes of interest. A & B) Technical validation of our candidate CpG sites or DMRs (Benign CP n=6, TAN GS6 n = 6, TAN GS89 = 5). C & D) Independent validation of KRTAP27-1 and methylation analysis of APC, GSTP1, HOXD3 and RASSF1A. PMR values represent the level of methylation for each gene. (APC, GSTP1, RASSF1A, HOXD3: Benign CP n ≤ 17, TAN n ≤ 66; KRTAP27-1: Benign CP n = 12, TAN GS6 n = 25, TAN GS7 n = 34).
2.3.5 Independent validation of KRTAP27-1 using MethyLight Technology

In addition to these novel genes, we decided to measure the methylation patterns of known/established gene methylation markers HOXD3, APC, GSTP1 and RASSF1A in all samples analyzed by the array and from Cohort 2 that consists of TAN (up to 66 samples) and benign CP (up to 17 samples). Except for HOXD3, methylation of these known genes in TAN tissue has been reported in the literature\textsuperscript{155,157,165}. As mentioned previously, APC, GSTP1 and RASSF1A constitute the gene panel in the ConfirmMDx assay. We observed a significant increase in the methylation of RASSF1A in TAN cases (n = 64) compared to benign cases (n = 16) (Figure 2.5 C, p = 0.002). When TAN cases were stratified by GS, HOXD3 and RASSF1A methylation was significantly elevated in TAN GS 6 cases (Figure 2.5 D, n = 30, p = 0.007 and n=31, p = 0.004, respectively), while RASSF1A also showed significantly higher methylation in TAN GS 7 cases (Figure 2.5 D, n = 33, p = 0.007). An increasing trend of APC methylation in TAN GS 6 cases (n = 34) was also observed. However, the difference was not statistically significant (Figure 2.5 D, p = 0.066). Surprisingly, there was a significant decrease in GSTP1 methylation in TAN GS 6 compared to benign CP cases (Figure 2.5 D, p = 0.04).

Independent validation of KRTAP27-1 was performed on Cohort 2, which included benign CP cases (n = 12) and TAN cases (n = 59) from patients with low- (GS 6, n = 25) and intermediate- (GS 7, n= 34) grade PCa that were not analyzed on the array. Comparative analysis revealed no difference between methylation patterns of KRTAP27-1 in benign CP vs. TAN cases in this cohort (Figure 2.5 C&D, p > 0.05). When stratified based on Gleason score, the difference is not statistically significant.

2.4 Discussion

In this chapter, we established that TAN prostate tissue at a distance of up to 8 mm away from the tumour focus harbours DNA methylation changes that can be detected using methylation-specific qPCR. APC and RASSF1A displayed similar methylation profiles between TAN prostate tissue 0-4 and 4-8 mm away from a tumour. In contrast, GSTP1 methylation significantly decreased at increasing distances away from the tumour. Most importantly, these results revealed that APC, RASSF1A and GSTP1 are methylated in both tumour and TAN
tissues, despite the significant difference in methylation levels. These findings suggest that negative biopsy tissue taken within this distance from a tumour focus may harbour differentially methylated genes that can potentially be used for clinical diagnosis of PCa, even when the histologic diagnosis does not show evidence for cancer.

Due to the limited size of the RP specimens examined, we were unable to obtain enough cases with TAN areas greater than 8 mm from a tumour. Beyond this distance, it is possible that the presence of other tumours can influence then methylation signals for our genes of interest. Furthermore, our TAN cases were micro dissected from 2-dimensional RP specimens. Other tumours could also be present above or below the interrogated region in 3-dimensional space, influencing the methylation patterns we observed in the TAN tissues. Although we were able to detect DNA methylation alterations up to 8 mm away from a tumour, this may be caused by field cancerization surrounding more than one tumour.

We also observed a distinct methylation signature that separates TAN and benign CP prostate tissue. This provides evidence of field cancerization altering DNA methylation patterns in TAN prostate tissue. Three different analytical approaches were used to identify 8 differentially methylated CpG sites and regions to ensure that the most robust candidate biomarkers would be identified. A total of 5 CpG sites and 3 DMRs were identified based on stringent criteria mentioned above. Each CpG site of interest was associated with one gene. Both KRTAP3-3 and KRTAP27-1 encode Keratin-associated proteins that are involved in maintaining the structural integrity of hair cells\textsuperscript{177}. HSD3B2 (hydroxyl-delta-5-steroid dehydrogenase) encodes a protein that converts cholesterol to progesterone\textsuperscript{178,179}. The function of both KLHDC7A (Kelch-domain-containing protein 7A family member) and C5orf64 has not been characterized. Three DMRs were identified using Bump hunting analysis. However, due to the inability to design reliable primers and probes for qPCR analysis of MYO15B and an uncharacterized DMR, we performed technical validation of VTRNA2-1 only. VTRNA2-1 is a non-coding RNA that functions as a tumour suppressor and represses cellular Protein Kinase RNA and indirectly promotes cell death\textsuperscript{180–182}. A region containing 8 CpG sites located in the promoter region of VTRNA2-1 was significantly hypermethylated in TAN GS6 cases. The function of MYO15B and the uncharacterized DMR are unknown. Understanding the functional purpose of genes differentially methylated in TAN tissues could suggest how their methylation pattern may
be affecting their expression and contributing to PCa development. However, genes that are differentially methylated in TAN tissues that can be used to identify PCa patients are most relevant, regardless of their biological function.

Our inability to validate VTRNA2-1 and KRTAP3-3 methylation using methylation-specific qPCR could be due to a number of reasons. Firstly, the primer and probe design often requires binding to additional CpG sites near our candidate CpG to allow for optimal amplification of the region. Consequently, the methylation levels of these additional CpG sites may have affected the overall methylation patterns we observed between groups using MethyLight. Secondly, technical validation was performed on a relatively small number of array cases (n = 19). As a result, the variability in methylation levels between samples greatly influenced the results of our analysis. A larger cohort of samples is necessary to minimize the effects of variability and accurately compare the methylation patterns between benign CP cases and TAN cases.

Although the methylation of KLHDC7A in TAN cases was significantly higher compared to benign CP cases, the overall methylation levels for both groups were very high (PMR > 100%). This may be due to incomplete methylation of super-methylated DNA (positive control), causing highly methylated genes to have PMR values over 100%\textsuperscript{173}. If used as a biomarker, it may be difficult to identify patients with PCa if non-cancer patients also exhibit high methylation of this CpG site. Therefore, only the independent validation of KRTAP27-1 was performed on Cohort 2 using MethyLight technology. Since we are interested in investigating its potential as a biomarker for early and accurate PCa diagnosis, we only analyzed benign, TAN GS6 and TAN GS7 cases in our independent cohort. Methylation patterns of known genes (GSTP1, RASSF1A, HOXD3 and APC) were also analyzed in both the array cohort and Cohort 2. Since methylation of these genes has previously been reported in TAN or matched normal prostate tissues\textsuperscript{155,157,165}, detecting similar methylation patterns in our cohorts can confirm the validity of our biomarker validation method. Our independent validation of KRTAP27-1 methylation revealed comparable methylation levels between benign CP and TAN cases taken together and stratified by GS. This may be due to the difference in the proportion of TAN cases studied in this cohort. In the array and the technical validation, TAN tissue was taken from patients with GS 6, GS 8 and 9 tumours. However, Cohort 2 consisted of TAN tissue from patients with GS 6 and GS 7 tumours. The differences in the two cohorts may have contributed
to discordant results observed in our study. Furthermore, we observed high KRTAP27-1 methylation in the benign CP cases. Interestingly, GSTP1 methylation was also significantly elevated in benign CP cases compared to TAN GS 6 cases. This was also reported in a study performed by Kwabi-Abdo et al. (2007)\textsuperscript{183}. Therefore, we speculate that the elevated methylation pattern of GSTP1 and KRTAP27-1 in benign CP cases may be due to the abnormal pathology of each patient within the benign CP group.

Cases included in the benign CP cohort were microdissected from cystoprostatectomy specimens obtained from patients who have high-grade urothelial carcinoma, although the prostate tissue remains histologically normal. Since organs surrounding the prostate have evidence of carcinoma, it is possible that the benign CP prostate tissue analyzed may also be affected by field cancerization, altering the DNA methylation profile within benign prostate tissue. For instance, none of the CpG sites interrogated on the array were significantly differentially methylated according to LIMMA and methyAnalysis when using adjusted p-value criteria (corrected for multiple comparisons) to filter candidate genes. Therefore, we were limited to the use of unadjusted p-values to identify differentially methylated CpG sites or regions. It is possible that the methylation patterns of these benign CP cases may not reflect the true methylation profile of healthy prostate tissue. As a result, the extent of differential methylation observed between groups is subtle and revealed higher-than-expected methylation of KRTAP27-1 and GSTP1 in the benign CP cohort. Unfortunately, it is difficult to obtain normal prostate tissue from healthy subjects and remains a limitation of this project.

Future studies could be performed using pyrosequencing or bisulfite sequencing strategies for technical and independent validation of novel genes identified using the Illumina 450K methylation microarray\textsuperscript{184–186}. Both of these methods can interrogate and measure the methylation pattern of a specific CpG site and consequently, provide more accurate validation results. Also, the use of healthy prostate tissue from healthy male donors may serve as a better control group and provide a more accurate comparison between cancer and non-cancer associated normal tissue. Unfortunately, these specimens may be more difficult to come by. Furthermore, our CpG site of interest associated with KRTAP27-1 was identified based on significant hypermethylation observed in a cohort of TAN cases surrounding low (GS6) and high (GS89) grade tumours. Independent validation using a larger series with TAN cases
surrounding low (GS6) and high (GS89) risk tumours and normal prostate tissue from healthy patients may confirm the association between PCa and KRTAP27-1 hypermethylation at our CpG site of interest.
Chapter 3: Evaluating the Methylation of Novel and Known genes in Normal, Tumour-Adjacent Normal and Tumour Prostate Tissue
3.1 Introduction

In the previous chapter, we identified and validated the hypermethylation of a CpG site within an exon of the KRTAP27-1 gene. Although independent validation revealed similar methylation patterns between TAN and benign CP cases, hypermethylation of this gene may be useful as a diagnostic marker if used in combination with other genes. Many groups have developed diagnostic tests that combine multiple biomarkers to improve the detection of PCa\textsuperscript{143–146}. For example, the 4K score measures the levels of tPSA, fPSA, intact PSA and human kallikrein 2 and calculates a score that reflects the probability of detecting high-risk PCa upon initial or repeat biopsy\textsuperscript{144,145}. Similarly, Mi-PS test combines TMPRSS2:ERG, PCA3 and PSA transcript levels, while the PHI test combines tPSA, %fPSA and -2proPSA to evaluate the risk of having PCa based on strong evidence that these biomarkers perform optimally as a panel for PCa detection\textsuperscript{143,146}. Unfortunately, none of these diagnostic tests can predict the presence of PCa in patients who received an initial negative biopsy result. Therefore, we evaluate the methylation of our genes individually and in combination to determine their efficacy as potential biomarkers for PCa detection in negative biopsy tissue.

3.1.1 Selecting Potential Diagnostic Biomarkers for PCa Detection

Genes reported to be hypermethylated in PCa that can discriminate between PCa and healthy patients are the most promising diagnostic biomarkers for PCa detection. APC is a tumour suppressor gene that functions as a regulator of the Wnt signalling pathway through degradation of beta-catenin\textsuperscript{124}. Similarly, RASSF1A is also a tumour suppressor gene that regulates cell cycle progression by controlling the transition from G1 to S phase\textsuperscript{116}. Although both genes are known to be hypermethylated in PCa tumours, they are currently used as field cancerization biomarkers to discriminate between PCa and non-PCa patients in negative biopsy tissues\textsuperscript{161}. This suggests that other differentially methylated genes in prostate tumours may also be useful as field cancerization biomarkers for PCa detection. In this chapter, we analyze the methylation of KRTAP27-1, HOXD3, KLK6 and CRIP3 in addition to APC and RASSF1A to evaluate their efficacy individually and in combination as field cancerization biomarkers to improve PCa diagnosis in negative biopsy tissue.
KRTAP27-1 was the most promising biomarker identified in our discovery analysis between TAN and benign CP tissues. Using two separate techniques, we were able to verify the differential methylation results between the two tissues. Therefore, we decided to further examine the methylation of KRTAP27-1 in combination with other potential diagnostic biomarkers. HOXD3 and KLK6 were also selected for further investigation as field cancerization biomarkers due to previous evidence of their ability to distinguish between benign and PCa patients\textsuperscript{165,187}. HOXD3 is a transcription factor that is a part of the Homeobox (HOX) gene family\textsuperscript{188}. Previous studies in our lab revealed that HOXD3 is significantly hypermethylated in tumour vs. matched normal prostate tissue and may have prognostic value in identifying patients with BCR\textsuperscript{165}. Additional studies in our lab also revealed that KLK6 methylation is elevated in tumours compared to matched normal prostate tissue\textsuperscript{187}. KLK6 is a serine protease that is part of the Kalikrein family of genes. Interestingly, a positive association between tumour and matched normal methylation was also observed, which suggests that KLK6 methylation may be associated with field cancerization. Although differential methylation was observed between tumour and TAN prostate tissue, these genes have not been studied in the context of field cancerization and may have potential roles as biomarkers for PCa detection in negative biopsy tissue.

Our lab previously discovered hypermethylation of CRIP3 in prostate tumours and observed its progressive association with increasing stages of PCa. The biological function of CRIP3 is currently unknown. Recently, our lab investigated CRIP3 methylation in post-DRE urinary sediments from low risk (GS 6) PCa patients monitored by Active Surveillance\textsuperscript{189}. CRIP3 methylation in combination with methylation of APC, GSTP1 and HOXD8 in urinary sediments of these patients shows promise as a biomarker classifier panel to predict reclassification of PCa patients for increased risk of cancer progression. This is the first publication that revealed the significance of CRIP3 methylation as a PCa biomarker. However, no studies have been conducted to evaluate CRIP3 methylation in the context of field cancerization. Therefore, we were interested in investigating CRIP3 methylation in tumour, TAN and benign prostate tissue to determine if it has diagnostic value for PCa detection in combination with other genes of interest.
3.1.2 Differential Methylation in Tumour and TAN Prostate Tissue

Tumour and TAN prostate tissues have been reported to share similar DNA methylation patterns compared to tumour and normal prostate tissue from healthy patients\textsuperscript{163}. Two studies have revealed that many of the genes that were differentially methylated in tumour tissues were also found in TAN tissues, but absent in normal prostate tissue\textsuperscript{127,163}. This provides strong evidence that field cancerization that promotes tumour development also alters the methylation profile in TAN tissue in a similar fashion. Genes found to be differentially methylated in both tumour and TAN tissues would be ideal biomarkers for PCa detection in cancer-negative biopsy tissue. In this chapter, we follow our Biomarker Investigation Model (Figure 3.1) to evaluate the concordance of methylation between matched tumour and TAN tissue for our genes of interest. This will reveal their feasibility as DNA methylation biomarkers for PCa detection. To identify which genes will have the best performance in detecting PCa patients with initial negative biopsies, additional methylation analysis was performed between TAN and benign CP prostate tissue for our genes of interest.
3.2 Materials and Methods

3.2.1 Patient Cohort

Matched RP and Biopsy (Bx) prostate tissue obtained from up to 152 patients per gene (range: 28-152) were analyzed for the methylation of our candidate genes. Tumour and TAN prostate tissue from RP and Bx specimens were microdissected for DNA methylation analysis. The concordance of methylation was analyzed between the following groups: matched RP tumour and TAN prostate tissue, matched RP and Bx tumour prostate tissue, and matched RP tumour and Bx TAN prostate tissue (Figure 3.1). Methylation was also analyzed between patients with either RP or Bx TAN and benign CP prostate tissue to develop a cut off that ensured 100% specificity. Methylation of selected genes was compared between three groups: up to 72 RP TAN, 40 Bx TAN cases and 17 benign CP cases were compared. All patients were recruited from the University Health Network (Toronto, Canada) with REB approval. Due to the limited availability and/or quality of DNA, not all genes were analyzed for the same number of cases.

3.2.2 DNA Extraction, Bisulfite Modification and Quantitative, Methylation-Specific PCR

Benign CP, RP and Bx specimens from patients were obtained from FFPE blocks and cut into 5-10 um serial sections for microdissection and DNA extraction. Samples within the benign CP tissue cohort are the same cases evaluated in Chapter 2. RP TAN cohort is comprised of a combination of all RP TAN cases analyzed in Chapter 2 and additional RP cases evaluated for TAN methylation by previous members of the lab. H&E slides were prepared for each block and were marked for normal, TAN and/or tumour tissue by Dr. T. van der Kwast, Dr. F. Siadat and/or Dr. S. Bellur. Marked H&E slides were superimposed over the corresponding unstained sections of benign CP, RP or Bx tissue to outline areas for DNA extraction. Method of extraction and bisulfite modification is described in the Materials and Methods section of Chapter 2. To measure the methylation of our genes of interest, we performed Methylight technology. Assay protocol is described in Chapter 2. Primer and probe sequences for APC, RASSF1A, HOXD3, CRIP3, KLK6 and KRTAP27-1 are listed in Table 2.1. Percent Methylated Reference (PMR) values were calculated to quantify the methylation of our genes of interest within each DNA sample. PMR values were calculated as follows: [Gene of
interest/ALU fluorescence quantity ratio for sample DNA)/(Gene of interest/ALU fluorescence quantity ratio for super methylated DNA)] x 100%. Each DNA sample was analyzed in either duplicate or triplicate.

3.2.3 Statistical Analysis

To examine the feasibility of our genes as field cancerization biomarkers for PCa detection, concordance of methylation between matched TAN and tumour cases was evaluated. A gene with a PMR > 0 was considered methylated, while a gene with a PMR = 0 was considered unmethylated. Quantitative methylation concordance of an individual gene or a combination of genes is described as the percentage of matched samples concordant in groups being compared. If both matched samples are methylated, they have concordant methylation. If one sample is methylated while the other sample is unmethylated, they have discordant methylation. Sensitivity of our genes to detect PCa cases was calculated using only matched Bx TAN cases from strictly PCa patients based on the number of true positive cases (concordant methylation) and false negative cases (discordant methylation). Pearson correlation analysis was also performed to determine the association of methylation between groups.

To evaluate the performance of our genes to accurately detect PCa patients, we analyzed the methylation patterns between benign CP and RP or Bx TAN prostate tissue. Receiver Operating Characteristic (ROC) analysis was conducted to determine the cut off value for each gene that ensured 100% specificity. Cases with a PMR above the cut off were considered to be hypermethylated. Multi-gene analysis models were performed to identify combinations of genes that can improve the predictive ability over any one gene. When a case is above the cut off value of at least one gene included in the multi-gene panel, they were considered to be hypermethylated. Some cases included in a paired or multi-gene panel may have methylation analyzed for only some of the genes. These cases were included only if 1 or more genes were hypermethylated. However, if none of the genes had hypermethylation, they were excluded from the analysis. Chi-squared ($X^2$) analysis was performed to determine the association of gene hypermethylation with PCa for any gene individually, or genes in paired or multi-gene panels. Area under the curve (AUC), sensitivity, specificity, Positive Predictive Value (PPV) and NPV
were also evaluated for all individual or multi-gene models. Statistical methods have also been performed previously\textsuperscript{126}.

3.3 Results

3.3.1 Establishing the Quantitative Concordance of Methylation Between Matched Tumour and TAN Prostate Tissue.

Matched RP tumour and either RP or Bx TAN prostate tissue were analyzed for the methylation of APC, RASSF1A, HOXD3, CRIP3, KLK6 and KRTAP27-1 (Figure 3.1). Approximately 82\% of all patients had methylation detected in both RP tumour and RP TAN cases for 1 or more genes (Table 3.1). KLK6 had the highest concordance of methylation (94.7\%), while APC had the lowest concordance of methylation (60\%). A lower overall concordance of methylation was observed between RP tumour and Bx TAN tissues (66\%, Table 3.1). RASSF1A had the highest concordance of methylation between both tissue types (88\%), while APC had the least concordance of methylation between tissue types (42\%). As proof of principle, we also analyzed the concordance of methylation between matched RP and Bx tumour tissue for APC and HOXD3 (Table 3.1). As a result, we observed that 93\% of patients had methylation concordance of APC and HOXD3 between matched RP and Bx tumour prostate tissue. More specifically, 100\% of all patients had HOXD3 methylation in both RP and Bx tumour tissue, while only 86\% of patients had APC methylation in both tissues.
Table 3.1. Concordance of methylation data between matched tumour and TAN prostate tissue.

*Out of the 82 Bx cases extracted for DNA, 45 Bx cases had sufficient quality to run on our genes. However, only up to 38 cases had matched RP Tumour and Bx TAN Methylation data for analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>RP Tumour vs. RP TAN Methylation</th>
<th>RP Tumour vs. Bx Tumour Methylation</th>
<th>RP Tumour vs. Bx TAN Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Concordance (%)</td>
<td>n</td>
</tr>
<tr>
<td>APC</td>
<td>78</td>
<td>60.3</td>
<td>14</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>138</td>
<td>83.9</td>
<td>-</td>
</tr>
<tr>
<td>HOXD3</td>
<td>131</td>
<td>81.7</td>
<td>14</td>
</tr>
<tr>
<td>CRIP3</td>
<td>130</td>
<td>76.2</td>
<td>-</td>
</tr>
<tr>
<td>KLK6</td>
<td>95</td>
<td>94.7</td>
<td>-</td>
</tr>
<tr>
<td>KRTAP27-1</td>
<td>46</td>
<td>87</td>
<td>-</td>
</tr>
<tr>
<td>Overall Concordance</td>
<td>662</td>
<td>81.8</td>
<td>28</td>
</tr>
</tbody>
</table>

3.3.2 Qualitative analysis of methylation patterns between matched tumour and TAN prostate tissue

The association between tumour and TAN prostate tissue methylation from RP or Bx tissue was evaluated to determine the qualitative concordance of methylation between matched samples from PCa patients. Due to the limited amount of DNA, each gene may have been run for a different number of matched cases. Interestingly, KRTAP27-1 and APC methylation in RP tumour and RP TAN tissue is positively correlated (n=46, p=6.99x10^{-6}, r=0.598 for KRTAP27-1, n=78, p=0.002, r=0.339 for APC) (Figure 3.2 A, C). This positive correlation was also confirmed between RP tumour and Bx TAN tissue for the same genes (n=38, p=0.001, r=0.529 for KRTAP27-1, n=28, p=0.002, r=0.561 for APC) (Figure 3.2 B, D). Notably, methylation of HOXD3 in tumour prostate tissue from RP and Bx also revealed a strong, positive correlation (Figure 3.2 E, n=14, p=0.007, r=0.688), while CRIP3 methylation in RP tumour and Bx TAN tissue revealed a weak, but positive correlation (Figure 3.2 F, n=34, p=0.038, r=0.358).
Figure 3.2. Correlation of methylation of genes of interest between RP Tumour vs. Bx Tumour, RP or Bx TAN. A) KRTAP27-1, n = 46. B) KRTAP27-1, n = 38. C) APC, n = 78. D) APC, n = 28. E) HOXD3, n = 14. F) CRIP3, n = 34.
To evaluate the predictive ability of our genes of interest to indicate the presence of PCa, we compared the sensitivity of APC, RASSF1A, CRIP3, HOXD3, KLK6 and KRTAP27-1 methylation in Bx TAN tissue from PCa patients (Table 3.2). Bx TAN cases that have a PMR > 0 for any gene indicated a true positive result. Bx TAN cases with PMR = 0 methylation for any gene indicated a false positive result. Multi-gene sensitivity was calculated based on the true positive and false negative result of 2 or more genes in combination. If one or more of the genes had a PMR > 0, the case was given a true positive result. Up to 40 Bx TAN cases were analyzed for any one gene. We observed that any gene paired with RASSF1A had stronger predictive ability to detect PCa than any one gene alone (Table 3.2, average paired sensitivity = 92%). Similarly, KLK6, KRTAP27-1 and CRIP3 also revealed a high sensitivity when paired with another gene (Table 3.2, Average paired sensitivity = 90%, 82% and 80%, respectively). When CRIP3, KLK6, KRTAP27-1 and RASSF1A are combined as a panel, 100% of all patients with PCa are predicted to have PCa.

Table 3.2. Sensitivity analysis of paired gene biomarker panels in Bx TAN cases from PCa patients only

<table>
<thead>
<tr>
<th>Gene</th>
<th>APC</th>
<th>RASSF1A</th>
<th>HOXD3</th>
<th>CRIP3</th>
<th>KLK6</th>
<th>KRTAP27-1</th>
<th>Average Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>N</td>
<td>-</td>
<td>27</td>
<td>28</td>
<td>27</td>
<td>28</td>
<td>77.9</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>-</td>
<td>85.2</td>
<td>75</td>
<td>70.4</td>
<td>85.7</td>
<td>73.1</td>
<td></td>
</tr>
<tr>
<td>RASSF1A</td>
<td>N</td>
<td>27</td>
<td>-</td>
<td>27</td>
<td>34</td>
<td>31</td>
<td>93.7</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>85.2</td>
<td>-</td>
<td>92.6</td>
<td>97.1</td>
<td>96.7</td>
<td>96.7</td>
<td></td>
</tr>
<tr>
<td>HOXD3</td>
<td>N</td>
<td>28</td>
<td>27</td>
<td>-</td>
<td>27</td>
<td>28</td>
<td>85.3</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>75</td>
<td>92.6</td>
<td>-</td>
<td>77.8</td>
<td>92.8</td>
<td>88.4</td>
<td></td>
</tr>
<tr>
<td>CRIP3</td>
<td>N</td>
<td>27</td>
<td>34</td>
<td>27</td>
<td>-</td>
<td>31</td>
<td>83.8</td>
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<tr>
<td>Sensitivity (%)</td>
<td>70.4</td>
<td>97.1</td>
<td>77.8</td>
<td>-</td>
<td>90.3</td>
<td>83.3</td>
<td></td>
</tr>
<tr>
<td>KLK6</td>
<td>N</td>
<td>28</td>
<td>31</td>
<td>28</td>
<td>31</td>
<td>-</td>
<td>90.5</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>85.7</td>
<td>96.7</td>
<td>92.8</td>
<td>90.3</td>
<td>-</td>
<td>87.1</td>
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<td>KRTAP27-1</td>
<td>N</td>
<td>26</td>
<td>30</td>
<td>26</td>
<td>30</td>
<td>31</td>
<td>85.7</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>73.1</td>
<td>96.7</td>
<td>88.4</td>
<td>83.3</td>
<td>87.1</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
3.3.4 Analyzing the detection ability of candidate gene methylation using benign CP and TAN prostate tissue

Methylation of our genes of interest was analyzed between benign CP (up to n = 16) and RP (up to n = 152) or Bx (up to n = 40) TAN cases to examine their efficacy as biomarkers for PCa detection. Genes were analyzed individually and in combination in order to determine which genes are most successful in identifying PCa patients and minimizing false negative results. Using ROC curve analysis, we selected a PMR cut off value for all genes that ensured 100% specificity.

Table 3.3. Individual gene methylation analysis between benign CP and TAN cases

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tissue Type</th>
<th>CRIP3</th>
<th>HOXD3</th>
<th>APC</th>
<th>RASSF1A</th>
<th>KRTAP27-1</th>
<th>KLK6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total NN (n)</td>
<td>RP TAN</td>
<td>16</td>
<td>16</td>
<td>15</td>
<td>16</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bx TAN</td>
<td>16</td>
<td>16</td>
<td>15</td>
<td>16</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>Total PCa Cases (n)</td>
<td>RP TAN</td>
<td>145</td>
<td>144</td>
<td>95</td>
<td>152</td>
<td>72</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bx TAN</td>
<td>34</td>
<td>28</td>
<td>28</td>
<td>34</td>
<td>40</td>
<td>44</td>
</tr>
<tr>
<td>True Positives (n)</td>
<td>RP TAN</td>
<td>56</td>
<td>57</td>
<td>30</td>
<td>66</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bx TAN</td>
<td>11</td>
<td>5</td>
<td>7</td>
<td>21</td>
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<tr>
<td>False Negatives (n)</td>
<td>RP TAN</td>
<td>89</td>
<td>87</td>
<td>65</td>
<td>86</td>
<td>62</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bx TAN</td>
<td>23</td>
<td>23</td>
<td>21</td>
<td>13</td>
<td>36</td>
<td>39</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>RP TAN</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bx TAN</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tr>
<tr>
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<td>RP TAN</td>
<td>38.6</td>
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<td>31.6</td>
<td>43.4</td>
<td>13.9</td>
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<tr>
<td></td>
<td>Bx TAN</td>
<td>32.4</td>
<td>17.9</td>
<td>25.0</td>
<td>61.8</td>
<td>10.0</td>
<td>11.4</td>
</tr>
<tr>
<td>NPV (%)</td>
<td>RP TAN</td>
<td>15.2</td>
<td>15.5</td>
<td>18.8</td>
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<td>20.5</td>
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<tr>
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<td>Bx TAN</td>
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<td>41.0</td>
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<td>PPV (%)</td>
<td>RP TAN</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
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<td>Bx TAN</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>100</td>
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<tr>
<td>P Value (X2)</td>
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<td>0.002</td>
<td>0.011</td>
<td>0.001</td>
<td>0.113</td>
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</tr>
<tr>
<td></td>
<td>Bx TAN</td>
<td>0.01</td>
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<td>0.034</td>
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<td>0.176</td>
<td>0.172</td>
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<tr>
<td>AUC</td>
<td>RP TAN</td>
<td>0.71</td>
<td>0.65</td>
<td>0.59</td>
<td>0.70</td>
<td>0.53</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bx TAN</td>
<td>0.55</td>
<td>0.52</td>
<td>0.20</td>
<td>0.62</td>
<td>0.48</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Initially, we compared the methylation patterns between benign CP and RP TAN cases. We observed that CRIP3 and RASSF1A were most successful in discriminating between PCa and non-PCa patients as an individual biomarker (AUC = 0.71 and 0.70, sensitivity = 39% and 43%, NPV = 15% and 15% respectively, Table 3.3, Figure 3.3 A). When we combined all genes into
paired biomarker panels, more PCa patients were accurately detected based on a higher sensitivity of paired vs. individual gene biomarkers (Table 3.4, Figure 3.3 B). Out of the 10 paired biomarker panels, RASSF1A with KRTAP27-1 and HOXD3 with APC hypermethylation were most significantly associated with PCa (p = 8.44 x 10^{-8} and 1.38 x 10^{-7}, respectively). Since these 2 paired gene panels showed the most promise as predictive biomarkers (RASSF1A with KRTAP27-1: AUC = 0.78, sensitivity = 67%, NPV = 29%; HOXD3 with APC: AUC = 0.79, sensitivity = 66%, NPV = 28%), we combined them to create a multi-gene biomarker panel. As a result, RASSF1A, KRTAP27-1, HOXD3 and APC in combination improved the PCa detection rate while minimizing false negative results (AUC = 0.90, sensitivity = 0.79, NPV = 0.52, p = 1.81 x 10^{-8}, Table 3.4, Figure 3.3 C).

Table 3.4. Paired and Multi-gene analysis of gene methylation in benign CP vs. RP TAN cases

<table>
<thead>
<tr>
<th>Gene combinations</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>NPV (%)</th>
<th>PPV (%)</th>
<th>P value (X^2)</th>
<th>Benign CP Cases (n)</th>
<th>PCa Cases (n)</th>
<th>True Positives (n)</th>
<th>False Negatives (n)</th>
<th>AUC</th>
</tr>
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<tbody>
<tr>
<td>HOXD3, RASSF1A and KRTAP27-1</td>
<td>79.5</td>
<td>100</td>
<td>36.8</td>
<td>100</td>
<td>5.87E-10</td>
<td>14</td>
<td>117</td>
<td>93</td>
<td>24</td>
<td>0.90</td>
</tr>
<tr>
<td>KRTAP27-1, RASSF1A, HOXD3 and APC</td>
<td>79.4</td>
<td>100</td>
<td>51.9</td>
<td>100</td>
<td>1.81E-08</td>
<td>14</td>
<td>63</td>
<td>50</td>
<td>13</td>
<td>0.90</td>
</tr>
<tr>
<td>RASSF1A and KRTA27-1</td>
<td>67.3</td>
<td>100</td>
<td>29.4</td>
<td>100</td>
<td>8.44E-08</td>
<td>15</td>
<td>110</td>
<td>74</td>
<td>36</td>
<td>0.78</td>
</tr>
<tr>
<td>CRIP3, KRTAP27-1 and RASSF1A</td>
<td>70.9</td>
<td>100</td>
<td>33.3</td>
<td>100</td>
<td>1.29E-07</td>
<td>15</td>
<td>103</td>
<td>73</td>
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</tr>
<tr>
<td>HOXD3 and APC</td>
<td>66.1</td>
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<td>27.8</td>
<td>100</td>
<td>1.38E-07</td>
<td>15</td>
<td>115</td>
<td>76</td>
<td>39</td>
<td>0.79</td>
</tr>
<tr>
<td>CRIP3 and KRTAP27-1</td>
<td>65.3</td>
<td>100</td>
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<td>100</td>
<td>2.76E-07</td>
<td>15</td>
<td>101</td>
<td>66</td>
<td>35</td>
<td>0.74</td>
</tr>
<tr>
<td>APC and RASSF1A</td>
<td>63.4</td>
<td>100</td>
<td>23.8</td>
<td>100</td>
<td>3.89E-07</td>
<td>15</td>
<td>131</td>
<td>83</td>
<td>48</td>
<td>0.84</td>
</tr>
<tr>
<td>CRIP3 and RASSF1A</td>
<td>62.3</td>
<td>100</td>
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<td>100</td>
<td>5.16E-07</td>
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<td>146</td>
<td>91</td>
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<td>0.82</td>
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<tr>
<td>CRIP3 and APC</td>
<td>63.2</td>
<td>100</td>
<td>25.9</td>
<td>100</td>
<td>5.18E-07</td>
<td>15</td>
<td>117</td>
<td>74</td>
<td>43</td>
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</tr>
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<td>HOXD3 and KRTAP27-1</td>
<td>61.9</td>
<td>100</td>
<td>27.3</td>
<td>100</td>
<td>1.17E-06</td>
<td>15</td>
<td>105</td>
<td>65</td>
<td>40</td>
<td>0.71</td>
</tr>
<tr>
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<td>100</td>
<td>21.9</td>
<td>100</td>
<td>1.43E-06</td>
<td>16</td>
<td>143</td>
<td>86</td>
<td>57</td>
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</tr>
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<td>100</td>
<td>20.5</td>
<td>100</td>
<td>1.49E-06</td>
<td>15</td>
<td>145</td>
<td>87</td>
<td>58</td>
<td>0.77</td>
</tr>
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<td>APC and KRTAP27-1</td>
<td>52.0</td>
<td>100</td>
<td>28.0</td>
<td>100</td>
<td>5.95E-05</td>
<td>14</td>
<td>75</td>
<td>39</td>
<td>36</td>
<td>0.76</td>
</tr>
</tbody>
</table>
Figure 3.3. ROC curves that reflect the performance of individual or multi-gene biomarker panels involving benign CP and RP TAN cases. A) Individual gene biomarkers. Number of cases analyzed per gene, AUC and sensitivity statistics reported in Table 3.3. B) Paired gene biomarker panels. Number of cases analyzed, AUC and sensitivity statistics reported in Table 3.4. C) Multi-gene biomarker panels. Number of cases analyzed per gene, AUC and sensitivity statistics reported in Table 3.4.

Likewise, we also compared the methylation patterns between benign CP and Bx TAN cases. CRIP3 and RASSF1A methylation also had the strongest predictive ability as individual biomarkers based on AUC (AUC = 0.55 and 0.62, sensitivity = 32% and 62%, NPV = 41% and 55% respectively, Table 3.3, Figure 3.4 A). When genes were paired, any gene combined with RASSF1A revealed a highly significant association with PCa (Range p = 2.08 x 10^-6 to 4.32 x 10^-5) compared to other paired gene models. Furthermore, RASSF1A paired with either KRTAP27-1, CRIP3 or HOXD3 had the greatest performance in identifying PCa patients (AUC = 0.82, 0.86 and 0.84, sensitivity = 72%, 74% and 70%, NPV = 64%, 63% and 63%, respectively, Table 3.5, Figure 3.4 B). Combining all four genes into a multi-gene biomarker
panel improved the detection of PCa cases compared to the other gene models (AUC = 0.89, sensitivity = 79% and NPV = 68%, p = 3.82 x 10^{-7}, Table 3.5, Figure 3.4 C).

Table 3.5. Paired and Multi-gene analysis of gene methylation in benign CP vs. Bx TAN cases.

<table>
<thead>
<tr>
<th>Gene combinations</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>NPV (%)</th>
<th>PPV (%)</th>
<th>P value (X^2)</th>
<th>Benign CP Cases (n)</th>
<th>PCa Cases (n)</th>
<th>True Positives (n)</th>
<th>False Negatives (n)</th>
<th>AUC</th>
</tr>
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<td>6</td>
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<td>68.2</td>
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<td>45.2</td>
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<td>45.7</td>
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<td>1.10E-02</td>
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<td>28</td>
<td>9</td>
<td>19</td>
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<tr>
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<td>44.1</td>
<td>100</td>
<td>1.40E-02</td>
<td>15</td>
<td>28</td>
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<tr>
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<td>9</td>
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</tr>
<tr>
<td>KRTAP27-1 &amp; KLK6</td>
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<td>32.6</td>
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<td>15</td>
<td>40</td>
<td>9</td>
<td>31</td>
<td>0.64</td>
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</table>

As mentioned above, we identified a 4-gene panel consisting of RASSF1A, CRIP3, KLK6 and KRTAP27-1 that detected all PCa patients based on Bx TAN methylation (PMR > 0) observed for at least one gene. We also analyzed the predictive ability of these genes using the cohorts that contain both benign CP and TAN cases. Since KLK6 was not analyzed for the same set of RP TAN cases, it was excluded from analysis between benign CP and RP TAN cases. We
observed that the multi-gene panel consisting of RASSF1A, CRIP3, KRTAP27-1 and KLK6 (for benign CP vs. Bx TAN cases only) performed optimally in both analysis involving either Bx or RP TAN cases. AUC, sensitivity and NPV were 0.85, 71% and 33%, respectively for the analysis involving benign CP vs. RP TAN cases (Table 3.4, Figure 3.3 C) and 0.91, 82% and 70% respectively, for the analysis involving benign CP vs. Bx TAN cases (Table 3.5, Figure 3.4 D). Additionally, we observed that RASSF1A, HOXD3, and KRTAP27-1 were identified as top candidate biomarkers that can best distinguish between benign and TAN cases when combined in paired or multi-gene panels (Table 3.4 and 3.5). When combined into a three-gene panel, their ability to identify PCa patients was comparable to or improved the performance of other multi-gene models that were investigated (Benign CP vs. RP TAN cases: AUC = 0.90, sensitivity = 79%, NPV = 37% (Table 3.4, Figure 3.3 C); Benign CP vs. Bx TAN cases: AUC = 0.93, sensitivity = 86%, NPV = 79% (Table 3.5, Figure 3.4 E).
Figure 3.4. ROC curves that reflect the performance of individual or multi-gene biomarker panels involving benign CP and Bx TAN cases. A) Individual gene biomarkers. Number of cases, AUC and sensitivity statistics reported in Table 3.2. B) Paired gene biomarker panels. Number of cases analyzed per gene, AUC and sensitivity statistics reported in Table 3.5. C-E) Multi-gene biomarker panels. Number of cases analyzed per gene, AUC and sensitivity statistics reported in Table 3.5.
3.4 Discussion

Previous studies have reported that the DNA methylation profiles of prostate tumours are more similar to DNA methylation profiles of matched TAN prostate tissue compared to normal prostate tissue from non-cancer patients due to field cancerization\(^{127,163}\). To confirm this finding, we determined the methylation concordance of APC, RASSF1A, KRTAP27-1, HOXD3, CRIP3 and KLK6 in tumour and TAN RP tissue. This allowed us to evaluate their feasibility as field cancerization biomarkers to indicate the presence of PCa using negative biopsy samples. We observed that out of all patients who had tumour methylation of any gene, 81\% had methylation of the same gene detected in TAN tissue. The high concordance of methylation between RP TAN and tumour prostate tissue confirms that both tissue types share a similar methylation profile. This reveals the potential role of our selected genes as field cancerization biomarkers to indicate the presence of a nearby tumour when methylation is detected in TAN tissue from negative biopsy cores.

It has been well established that APC, RASSF1A, HOXD3 and KLK6 are methylated in prostate tumours\(^{116,124,165,187}\). Therefore, it is likely that a high concordance of methylation of our genes would be observed between tumour tissues from RP and matched pre-operative Bx specimens indicating a cancer diagnosis. As proof of principle, we examined the methylation of APC and HOXD3 in matched RP and Bx tumours and observed that 92\% of our patients had concordant methylation between tissues. This confirms that methylation of our genes is likely to be found in tumour tissue from either RP or Bx specimens. We decided not to analyze the methylation of other genes of interest due to limited tumour DNA sample availability. Revealing the concordance of methylation for all our genes between matched RP and Bx tumour tissue would not provide us supportive evidence to further test these genes for their potential as diagnostic biomarker in matched negative biopsy tissue.

When the methylation between RP tumours and Bx TAN cases was examined for our genes of interest, we observed an overall concordance of 66\%, with concordance of methylation varying for each individual gene (Range: 46.4-88.2\%). Compared to RP tumour and TAN prostate tissue, we observed a lower overall concordance of methylation between RP tumours and Bx TAN cases that could be explained by the multifocality of PCa. It was previously reported that
50.2% of patients with low risk PCa (GS 6) upgraded to intermediate or high-risk disease upon RP\textsuperscript{82}. This often occurs since many tumours exist within the prostate simultaneously and tumours found upon Bx are not necessarily obtained from the tumour foci within the prostate that has the highest GS. As a result, Bx TAN tissue that we examined could represent the methylation profile of different tumour foci than what was investigated on the RP specimen. Furthermore, the methylation of certain genes, such as APC, occurs at a lower frequency in TAN tissues compared to other genes, such as RASSF1A\textsuperscript{157,159}. This was confirmed in our analysis, where approximately 42% of cases with RP tumour methylation also had Bx TAN methylation of APC, compared to the 88% concordance of methylation observed for RASSF1A. Our results suggest that using a combination gene methylation panel in TAN tissue from Bx specimens may be more successful in detecting PCa.

We also investigated whether an association exists between prostate tumour and TAN methylation of RP and Bx tissues. Interestingly, both APC and KRTAP27-1 methylation in RP tumours was positively correlated with TAN methylation from both RP and Bx specimens. Similarly, a positive correlation was observed for CRIP3 methylation between RP tumour and Bx TAN tissue. This suggests that increased tumour methylation of APC, KRTAP27-1 and CRIP3 may also indicate increased levels of methylation in TAN tissue. Previous studies have reported that increased methylation of genes such as HOXD3 and APC are associated with high-grade PCa and have been implicated as predictive biomarkers for aggressive disease\textsuperscript{165,176}. If TAN and tumour methylation is qualitatively associated in genes implicated as prognostic biomarkers, they may be used to detect aggressive PCa using negative biopsy tissue. KRTAP27-1 and APC show the most promise as prognostic biomarkers since a positive association was observed between tumour and TAN prostate tissue. Furthermore, we also demonstrated that higher KRTAP27-1 TAN methylation was significantly associated with a higher GS using the array cases (Chapter 2, Figure 2.5 A & B), while an increasing trend in APC methylation was observed in TAN GS6 compared to benign CP cases (Chapter 2, Figure 2.5 D). Although we were unable to reproduce these results for KRTAP27-1 in our independent validation due to the TAN cohort composition (TAN GS 6 and TAN GS 7 cases), further analysis comparing KRTAP27-1 and APC TAN methylation between high and low risk PCa patients may reveal the potential role of these genes as predictive biomarkers for PCa aggressiveness.
To determine the efficacy of multiple genes in combination to detect PCa, we compared the sensitivity of 2 or more genes based on the presence or absence of methylation in Bx TAN tissue from PCa patients only. This revealed which genes can potentially be used as a biomarker panel to identify PCa in patients when found to be methylated in negative biopsy tissue. We observed that RASSF1A, KLK6, KRTAP27-1 and CRIP3 had the highest overall sensitivity when paired with any other gene (average paired sensitivity > 80%). Combined into a 4-gene biomarker panel, methylation of at least 1 gene in Bx TAN tissue was observed in all PCa patients (sensitivity = 100%). These results suggest that RASSF1A, KLK6, KRTAP27-1 and CRIP3 used in combination may successfully detect PCa in negative biopsy tissue affected by field cancerization. However, biomarker panels should also reveal high specificity, PPV and NPV to provide strong evidence so that they can be used successfully in a clinical setting.

When we analyzed the methylation of our genes between TAN and benign CP prostate tissue, we selected a cut off value that ensured 100% specificity. This allowed us to investigate the ability of individual or multi-gene panels to identify PCa after ensuring no false positive results (100% PPV). The ConfirmMDx assay can confidently rule out patients with no PCa if they do not receive a positive methylation result. However, the assay has a poor PPV (29%), causing potential false positive results. By selecting cut offs that ensured 100% specificity for each gene, we could identify a biomarker panel that can supplement the ConfirmMDx assay and minimize the amount of non-PCa patients from experiencing overtreatment.

Overall, we observed that genes combined into paired or multi-gene biomarker panels had greater predictive ability to detect PCa cases than any one gene. Between benign CP and RP TAN prostate tissue, the AUC, sensitivity and NPV improved when multiple genes were combined. Multi-gene biomarker panels consisting of HOXD3, RASSF1A and KRTAP27-1 or HOXD3, RASSF1A, APC and KRTAP27-1 revealed that their methylation was most significantly associated with PCa and had the highest AUC, sensitivity, and NPV compared to any other gene models. In our sensitivity analysis involving only Bx TAN cases from PCa patients, RASSF1A, KLK6, CRIP3 and KRTAP27-1 were able to identify all PCa when at least 1 gene was observed to have a PMR > 0 for all patients (sensitivity = 100%). Therefore, we also evaluated the performance of these genes in a cohort with both benign CP and RP TAN
prostate tissue methylation. Since KLK6 RP TAN cases are not the same cases evaluated for RASSF1A, CRIP3 and KRTAP27-1, KLK6 was excluded from the analysis. RASSF1A, CRIP3 and KRTAP27-1 biomarker panel also revealed strong predictive ability that revealed an AUC of 0.85 with a sensitivity and NPV of 71% and 33%, respectively. Although it wasn’t the best performing biomarker panel based on AUC, methylation of these genes combined was very significantly associated with PCa.

The same type of analysis was performed between benign CP and Bx TAN prostate tissue. Unfortunately, we did not have access to an additional benign patient cohort and decided to use the same cohort that was compared to the RP TAN cases. Similarly, we observed that paired or multi-gene biomarker panels outperformed the predictive ability of individual genes to detect PCa patients while minimizing false negative results. Notably, RASSF1A combined with any other gene in a paired model performed better any other paired gene biomarker panels. This confirmed previous studies that revealed RASSF1A methylation was a highly sensitive biomarker\textsuperscript{116,157,159}. However, when RASSF1A was combined with KLK6, CRIP3 and KRTAP27-1 into a multi-gene panel, this improved the predictive ability of RASSF1A alone or paired, revealing an AUC of 91% with a sensitivity and NPV of 82% and 70%, respectively.

Based on the results from both analyses, the paired biomarker panels with the strongest PCa detection ability included RASSF1A, KRTAP27-1 and HOXD3. When combined into a three-gene panel, we observed that the methylation of these genes were most significantly associated with PCa in both comparisons. Furthermore, they had very high AUC, sensitivity and NPV. Since this was observed in both comparisons, this combination of genes shows the most promise as a diagnostic biomarker panel to detect PCa patients.

The main limitation of this project is the absence of an additional negative control cohort of healthy patients with no PCa to analyze alongside Bx TAN methylation. In previous studies, cohorts are often separated into a training and test set to evaluate the efficacy of a cut off value in separating two groups accurately\textsuperscript{153,190,191}. Although we had two separate TAN cohorts (Bx and RP), we only had 1 cohort of benign CP cases that was used in both analyses. Therefore, we were unable to identify an optimal methylation cut off value for a specific gene or gene panel that could most accurately distinguish between PCa and non-PCa patients. Currently, we are in
the process of obtaining negative biopsy tissue from patients with at least 2 consecutive biopsy examinations and no evidence of PCa. This will allow us to create separate training and test cohorts to perform more comprehensive statistical analysis.

As mentioned in Chapter 2, the benign CP prostate tissue was taken from patients with high-grade urothelial carcinoma and may possess methylation patterns that indicate field cancerization. For example, we observed high methylation values for KRTAP27-1 and GSTP1 in benign CP tissue that may be associated with field cancerization. Since the same cohort of benign CP cases were used in our analysis with RP and Bx TAN tissue in this chapter, we selected high methylation cut off values for certain genes to ensure 100% specificity. This may have influenced the number of false negative results detected since many PCa cases with a lower PMR value fell below the cut off for specific genes. Obtaining negative prostate biopsy tissue from patients with no urothelial carcinoma may allow us to identify methylation cut off values that can more accurately discriminate between non-PCa and PCa patients and improve the NPV and sensitivity of our biomarker panels under investigation.

Other limitations involved the quality and quantity of Bx tissues that were available for methylation analysis. Microdissection and DNA extraction from FFPE Bx prostate tissue yielded very limited amounts of DNA, which made it difficult to analyze all 6 genes per Bx sample. This caused us to exclude GSTP1 in our Chapter 3 analysis since not enough DNA was available. Furthermore, different genes have a different number of TAN Bx cases analyzed. By having the same number of cases analyzed per gene or gene panel, a more direct comparison of their performance can be made since the number of cases analyzed affects our calculations for NPV and sensitivity. Furthermore, only a few of our genes could be multiplexed using Methylight technology. Therefore, more DNA was required to analyze the methylation of each gene on separate assays. To overcome this limitation, redesigning primers and probes for our genes of interest may allow multiple genes to be multiplexed optimally. However, this would require a shifting of the amplified region and could create incomparable results to our previous analysis.
Chapter 4: Summary of Main Findings and Future Directions
4.1 Summary of Main Findings

Although needle biopsy examinations are the gold standard procedure for PCa diagnosis, it is prone to sampling bias, causing approximately 25-30% of PCa patients to receive false negative results on their first biopsy\textsuperscript{76-78}. The commercially available ConfirmMDx assay was designed to address this dilemma. However, it is also limited by its sensitivity (68%), specificity (64%) and PPV (29%), which may lead to 30% false positive results\textsuperscript{159}. Consequently, many non-PCa patients may undergo repeat biopsy examinations. DNA methylation alterations in TAN tissue are promising biomarkers to identify PCa patients with an initial negative biopsy result since biopsy tissue from these patients are likely removed from TAN areas affected by field cancerization. Therefore, the goal of my thesis project was to investigate DNA methylation patterns in TAN tissue and identify differentially methylated genes that can improve PCa detection in patients who received an initial negative biopsy test.

We observed that APC, RASSF1A and GSTP1 methylation in TAN prostate tissue could be detected up to 8 mm away as a result of field cancerization. This suggests that negative biopsy cores taken up to 8 mm away from a tumour may harbour differential methylation patterns that can indicate the presence of PCa. We also observed a distinct methylation signature between TAN and benign CP prostate tissue, further confirming the influence of field cancerization to alter the DNA methylation pattern in TAN prostate tissue. Five significantly differentially methylated CpG sites and 3 DMRs were identified from our methylation array results and were selected for further validation as potential candidate diagnostic biomarkers.

We were able to verify the differential methylation patterns of KRTAP27-1 and KLHDC7A observed in the methylation array using Methylight technology. Methylight results revealed that both genes had significantly elevated TAN methylation compared to benign CP. When TAN cases were stratified by GS, KLHDC7A methylation was significantly higher in both TAN GS 6 and TAN GS 8 and 9 when combined compared to benign CP cases. Similarly, KRTAP27-1 methylation in TAN GS6 cases was significantly elevated compared to benign CP cases. Due to the high methylation of KLHDC7A in both TAN and benign CP cases, KRTAP27-1 was the only gene selected for independent validation. Interestingly, independent validation of KRTAP27-1 methylation was not concordant with the results from the array and technical validation. Despite the discordance, we pursued further investigation of KRTAP27-1
methylation to examine its potential as a biomarker for PCa diagnosis in combination with other genes.

In addition to KRTAP27-1, we evaluated APC, RASSF1A, HOXD3, CRIP3 and KLK6 methylation levels to determine the concordance of methylation between matched tumour and TAN prostate tissue and examine their diagnostic ability as PCa biomarkers. Overall concordance of methylation between RP tumour and RP TAN, Bx tumour and Bx TAN tissue was approximately 81.8%, 92.9% and 65.6%, respectively. KLK6 had the highest concordance of methylation between RP Tumour and TAN cases, while HOXD3 and RASSF1A had highest methylation concordance between RP tumour and Bx tumour and RP tumour and Bx TAN cases, respectively. Interestingly, KRTAP27-1 and APC revealed a significant positive correlation between RP tumour and RP TAN methylation and RP tumour and Bx TAN methylation. When RASSF1A, CRIP3, KLK6 and KRTAP27-1 were combined, at least one of the genes was methylated per case to identify all PCa patients in Bx TAN tissue.

To further examine the predictive ability of our candidate biomarkers, methylation of RASSF1A, CRIP3, KLK6, KRTAP27-1, APC and HOXD3 was also analyzed in a cohort containing benign CP and TAN prostate tissue from RP or Bx specimens. CRIP3 and RASSF1A individually outperformed the predictive ability of other genes to detect PCa patients when analyzed between benign CP and either RP or Bx TAN tissue. However, when genes are combined into paired or multi-gene biomarker panels, the sensitivity, AUC and NPV increased, improving the overall detection rate. A biomarker panel containing RASSF1A, CRIP3, KLK6 and KRTAP27-1 revealed strong predictive ability when hypermethylation of these genes were evaluated in combination. This confirmed our previous results that revealed strong sensitivity of these genes in combination to detect PCa patients using Bx TAN tissue only. Furthermore, hypermethylation of HOXD3, RASSF1A and KRTAP27-1 in combination was most significantly associated with PCa in both analyses. This multi-gene biomarker panel revealed a high AUC, sensitivity and NPV that was superior to other biomarker panels. In conclusion, this project revealed the clinical potential of using field cancerization biomarkers for PCa detection using negative biopsy tissue.
4.2 Future Directions

Moving forward, we intend to evaluate the efficacy of our biomarkers for both PCa detection and prognosis using an additional cohort of negative biopsy tissue from non-cancer patients who had at least two consecutive negative biopsies and no histological evidence of PCa. By including these cases, additional analysis can be performed to identify methylation cut off values that can detect and stratify PCa patients based on their risk of having aggressive disease. Methylation cut off values for each gene will be determined using a training cohort and will be applied to a test cohort to compare the performance of our biomarkers individually and in combination (paired or multi-gene biomarker panel) for PCa detection and risk stratification.

The most promising PCa diagnostic and prognostic biomarker panel identified using the method mentioned above will also be compared to existing screening (i.e. PSA, the ConfirmMDx panel of biomarkers (APC, RASSF1A and GSTP1), PCA3 score, PHI, 4Kscore) and prognostic (i.e. OncotypeDx, Prostarix test) tests used prior to RP. Furthermore, we can determine the additive value of combining our biomarker panel with these existing clinical tests to see if this may improve PCa detection and patient risk stratification while minimizing false-positive and false-negative results.

To further investigate the efficacy of our biomarker panel separately or in combination with other screening tests, we will assess their performance in a separate cohort of patients (validation cohort) that consist of PCa cases and benign controls. Prostate tissue examined for gene methylation will be obtained from biopsy cores, which closely reflects clinical practice for PCa diagnosis and early prognosis. Initial negative biopsy cores will be obtained from PCa cases who had an initial false-negative followed by a positive biopsy result. Similarly, true-negative biopsy cores will be obtained from benign controls who had at least 2 consecutive negative biopsy results and no evidence of PCa. Comparative analysis will be performed to determine if our biomarker panel can improve PCa detection and prognosis as a stand-alone test or in combination with other clinical tests. This analysis will confirm the benefit of using field cancerization biomarkers for clinical PCa detection and early prognosis in negative biopsy tissue. Long-term goal of these studies will be to translate these results into clinical practice.


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