PROGNOSTIC GENE SIGNATURE FOR PROSTATE CANCER

PROGNOSTIC GENE SIGNATURE FOR INTERMEDIATE RISK PROSTATE CANCER

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LAY ABSTRACT

The over-treatment of prostate cancer patients is a significant concern, as recent clinical trials has shown that it can lead to significant patient morbidity. Although the Gleason Scoring system is a powerful predictor of lethal or indolent disease, a significant proportion of men who present with early stage Gleason Score 7 tumours experience poorer prognosis than expected. The goal of this study is to develop and optimize a gene signature that can be utilized on Gleason Score 7, intermediate risk prostate cancer patients to differentiate them into good and poor outcome groups. We hypothesize that this signature will be able to accurately predict outcome in a separate retrospective cohort of prostate cancer patients. In short, our study hopes to provide proof-of-principle that through the use of gene signatures, it is possible to better differentiate prostate cancer patients into different outcome groups so that they may receive more appropriate treatment specific to their disease type.

ABSTRACT

The Gleason Score (GS) is a powerful predictor of outcome among prostate cancer patients. Patients with tumours graded with a GS of 2 to 6 have a much greater chance of survival compared to those with a GS of 8 to 10. A significant proportion (~40%) of men present with early stage GS 7 tumours (indicating intermediate risk) for whom prognosis is highly variable.

Three gene signatures were derived from publicly available gene expression profiles of prostate cancers from the Swedish Watchful Waiting cohort: 1) The Genomic Grade Index consisted of the top 24 genes discriminating between high (8, 9 & 10) and low (≤ 6) GS tumours, 2) The Lethal Gene Score consisted of the top 24 genes discriminating between lethal and indolent disease within GS 7 tumours only, and 3) The network-based gene signature consisted of 88 genes.

When these gene signatures were tested *in silico* on the gene expression profiles of GS 7 patients in both the SWW and the Mayo cohort, patients were stratified into high and low risk for recurrence. These results demonstrate that gene signatures are capable of differentiating low risk and high risk patients within GS 7 tumours.

The prognostic capacity of our gene signature will be tested prospectively in a retrospective collection of archived prostate cancer tissue blocks from a phase 3 clinical trial, and it is hypothesized that the patients can be stratified into good

and poor outcome groups. NanoString Technology will be used to quantify mRNA values for the signature genes on selected paraffin blocks. Expression values of candidate genes will be correlated with patients' long-term follow-up information to derive a clinically meaningful signature. Outcome will be defined as biochemical recurrence or metastatic event.

The goal of this study is to identify multiple genes whose expression could be formulated into a clinically applicable assay, the implementation of which could serve to better stratify intermediate risk prostate cancer patients for appropriate treatment.

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LIST OF ABBREVIATIONS AND SYMBOLS

ASTRO	American Society for Therapeutic Radiology and Oncology
bp	Base pairs
BCR	Biochemical Recurrence
CI	Confidence Interval
CTG	Clinical Trials Group
DASL	cDNA Mediated Annealing, Selection, Extension, and Ligation
DNA	Deoxyribonucleic Acid
DRE	Digital Rectal Exam
DYNC1I1	Dynein Cytoplasmic 1 Intermediate Chain 1
ERG	Erythroblast transformation-specific Related Gene
ERSPC	European Randomized Study of Screening for Prostate Cancer
ESC	Embryonic Stem Cells
FDA	Food and Drug Administration
FFPE	Formalin-fixed Paraffin Embedded
GGI	Genomic Grade Index
GS	Gleason Score
Gy	Grays
H&E	Hemotoxylin and Eosin
HR	Hazard Ratio
iPSC	Induced Pluripotent Stem Cells
ISUP	International Society of Urological Pathology

KCNN2	Potassium Calcium-Activated Channel Subfamily N member 2
LGS	Lethal Gene Score
M0	Module 0
M1	Module 1
M2	Module 2
M3	Module 3
M4	Module 4
M5	Module 5
M6	Module 6
M7	Module 7
M8	Module 8
M9	Module 9
MYC	Myelocytomatosis Oncogene Cellular Homolog
PAM	Prediction Analysis for Microarrays
PI3K	Phosphoinositide 3-kinase
PKB	Protein Kinase B
PSA	Prostate Specific Antigen
PTEN	Phosphatase and Tensin Homolog
PTTG1	Pituitary Tumour-Transforming 1
REB	Research Ethics Board
RIN	Ribonucleic Acid Integrity Number
RT-PCR	Reverse Transcription Polymerase Chain Reaction

RNA	Ribonucleic Acid
SWW	Swedish Watchful Waiting
TGF	Transforming Growth Factor
TMPRSS2	Transmembrane Protease Serine 2
TNM	Tumour-Node-Metastasis
VEGF	Vascular Endothelial Growth Factor

DECLARATION OF ACADEMIC ACHIEVEMENT

The following is a declaration that the content of the research in this document has been completed by Brian Li and recognizes the contributions of Dr. Anita Bane, Dr. Bekim Sadikovic, and Dr. Jean-Claude Cutz, in both the research process and the completion of the thesis. Brian Li was responsible for data collection, data analysis, and composition of the manuscript. Dr. Anita Bane contributed to the study design and assisted with data analysis and manuscript review. Dr. Bekim Sadikovic and Dr. Jean-Claude Cutz provided insightful advice and manuscript review.

INTRODUCTION

1.1 Epidemiology of Prostate Cancer

According to the most recent publication by Canadian Cancer Statistics, approximately 2 in 5 Canadians will develop cancer in their lifetime and about 1 in 4 will die of cancer. Prostate cancer is the most commonly diagnosed cancer in Canadian men as of 2015, accounting for roughly 23.9% of estimated new cancer cases. 10.1% of the estimated deaths due to cancer are expected to be from prostate cancer. This makes it the third most common cause of cancer death next to lung and colorectal cancer (Cancer Canada Statistics, 2015).

There are many risk factors for prostate cancer, some better understood and documented than others. For instance, age and race are well-known, endogenous risk factors. It has been consistently shown that prostate cancer is seldom diagnosed in men younger than 50 years old (the average age of diagnosis being 67) and the highest incidence rates belong to African-American men (Siegel, Miller, & Jemal, 2015; Bostwick et al., 2004). Family history is also an important, well-established risk factor to consider, as first-degree relatives of men with prostate cancer have twice the risk of developing prostate cancer compared to the general population (Siegel, Miller, & Jemal, 2015; Eeles et al., 2014). Exogenous factors such as diet and environmental agents which can disrupt androgen homeostasis are also thought to be risk factors, though results from various studies have been inconsistent (Bostwick et al., 2004). Ultimately, prostate cancer poses a significant burden to men's health and the need to both

identify and mitigate risk factors is an important part of reducing the future prevalence of prostate cancer.

1.2 Prostate Cancer

There are a number of histological subtypes of prostate cancer, the most common being prostatic adenocarcinoma which is defined as cancer of epithelial cells originating from glandular tissue. Prostate cancer patients are typically categorized into low, intermediate, or high risk for poor outcomes such as recurrence and death. Clinical factors such as Gleason Score (GS), serum prostate-specific antigen (PSA), and tumour stage help determine which risk group patients should fall under. Patients diagnosed with low-risk disease can be safely placed under watchful waiting, while those diagnosed with high-risk disease are candidates for more aggressive treatments such as radical radiotherapy, adjuvant hormonal therapy, or radical prostatectomy. Determining a prostate cancer patient's risk status using clinical models is a relatively straightforward task, but does not always predict long-term outcome (Sboner et al., 2010; Markert, Mizuno, Vazquez & Levine, 2011). This is especially the case in patients diagnosed with intermediate risk disease, which will be the focus of this study. The uncertainty of long-term outcome is problematic because it may lead to patients and healthcare providers opting for aggressive therapies when in actuality, a more conservative approach would have been more appropriate. Over-treatment of indolent prostate cancer is a significant concern, as clinical trials have shown that up to 48 prostate cancer patients must be treated in order to

prevent a single prostate cancer related death (Schröder et al., 2009). Preventing over-treatment is difficult due to our limited ability to differentiate between indolent and aggressive tumours at the time of diagnosis, despite our established clinical methods. As mentioned above, there are three main predictive markers for prostate cancer outcome: tumour grade or GS, PSA levels, and tumour stage. Each marker is described below.

1.3 PSA Testing

PSA is a serine protease secreted by the prostate in order to liquefy seminal fluid by digesting clotting proteins released by the seminal vesicles (Lilja, Ulmert, & Vickers, 2008). It is expressed by the KLK3 gene, which is located on human chromosome 19q13.3-13.4, a region that has been associated with prostate cancer risk (Cicek, Liu, Casey, & Witte, 2005).

It is normal for trace amounts of PSA to be found in the blood where it holds no catalytic activity due to the presence of protease inhibitors. Changes in the prostate's size, architecture due to aging, infections, benign prostate diseases or cancer can cause excess PSA to leak into the circulatory system (Helfand et al., 2013). For this reason, PSA screening is a tool used, in conjunction with other clinical factors, to indicate the presence of prostate abnormality and guide prostate cancer management. It is important to recognize the utility of PSA as a marker of prostate cancer, but also realize that there are confounding factors to using this marker. Normal levels of serum PSA are considered to be between 1.0 and 4.0

ng/ml, though studies have shown that advanced stages of prostate cancer can be found within normal values and, conversely, the absence of prostate cancer can be found in above-normal PSA values (Thompson et al., 2004). Low levels of PSA in advanced stages of prostate cancer are relatively rare (Lee et al., 2010; Leibovici et al., 2007).

Elevated PSA levels can also be used as an indication of prostate cancer recurrence after treatment, which is referred to as an event called biochemical failure or biochemical recurrence (BCR). Although rising PSA levels after treatment are an established indication for recurrence, there are several definitions to describe BCR, which all vary on the threshold PSA value obtained one month after radical prostatectomy and the number of subsequent rises in PSA thereafter (Lilja, Ulmert & Vickers, 2008; Stephenson et al., 2006). A well-known and accepted definition for BCR was created by the American Society for Therapeutic Radiology and Oncology (ASTRO). In 1996, ASTRO held a consensus conference to generate a definition of BCR after external beam radiotherapy. The ASTRO definition states that PSA failure occurs after three consecutive PSA rises, with a failure date defined as the midpoint between the dates of the last nonmeasureable PSA value to the first rise great enough to provoke therapy (R.K. Institute, 1997). Houston and Vancouver definitions differ in elements such as the number of consecutive PSA rises post-nadir and the amount of time allowable between each rise (Pickles, Kim-Sing, Morris, Tyldesley, & Paltiel, 2003). A large randomized trial called the European Randomized Study of Screening for

Prostate Cancer (ERSPC) conducted in the 1990's investigated the effects of PSA testing on mortality rates from prostate cancer in 162,243 men. They found that the rate of prostate cancer death fell by 20% following the introduction of PSA screening, but was associated with a risk of over-diagnosis (Schröder et al., 2009). At this time, PSA testing is not currently recommended in Canada as a population-based screening test (Canadian Cancer Statistics, 2015).

1.4 Gleason Score

The Gleason Score (GS) was invented by Donald F. Gleason, a pathologist in the 1960's, in order to histologically grade prostatic carcinoma and predict patient prognosis (Gleason & Mellinger, 1974). A diagram of the original and current GS patterns is shown in **Figure 1**. Gleason pattern 1 refers to well differentiated, closely packed, uniform, medium-sized acini or glands, resembling normal prostate tissue. Gleason pattern 2 resembles Gleason pattern 1, though glands are more loosely arranged with minimal infiltration at the edge of the tumour nodule. Gleason pattern 3 consists of glands with ragged, poorly defined edges that are usually irregularly separated. Gleason pattern 4 includes fused microacinar glands, ill-defined glands and now include cribriform and glomeruloid patterns (as per the 2014 ISUP update). Gleason pattern 5 shows highly undifferentiated glands with central necrosis surrounded by papillary, cribriform, or solid masses.

The Gleason Scoring system works by adding together the most dominant pattern with the second most dominant pattern to yield a GS between 2 and 10. It is denoted as the primary pattern + secondary pattern, for instance, GS (3 + 5) 8. If only one pattern is found, it is deemed both the primary and secondary pattern. For example, a tumour containing only pattern 4 would be given a GS of (4 + 4) 8 resulting in a tumour classified as GS 8 (Epstein et al., 2005).

A tumour given a score between 2 and 6 is considered to be low risk for poor outcome, whereas a tumour given a score between 8 and 10 is considered to be high risk for poor outcome. A GS of 7 is classified as intermediate risk and can be further classified into GS 3 + 4 or 4 + 3. Although the GS has proven to be highly reliable in terms of recognizing tumour heterogeneity, it is limited in that it does not account for the multifocality of prostate cancer. Different tumour regions can have GS's that do not agree with the overall score. Despite this fact, many studies base their information on the largest tumour foci (Arora et al., 2004). In an untreated cohort of early stage prostate cancer patients, those diagnosed with GS 6 had a 10 year survival of approximately 80% while patients with GS 8 or higher had a 10 year survival of 20-30%. Patients with GS 7 tumours had a 10 year survival of approximately 60% (**Figure 7**) (Sboner et al., 2010).

In 2005, the International Society of Urological Pathology (ISUP) held a consensus conference on the Gleason Grading System in order to address inconsistencies and controversies that had arisen over the decades (Epstein et al., 2005). A schematic of the 2005 changes can be found in **Figure 2**. These changes

were based on the increase in radical prostatectomy specimens which require the grading of multiple nodules within the same prostate, the current use of immunohistochemical staining, and newly described variants of adenocarcinoma such as ductal adenocarcinoma or pseudohyperplastic adenocarcinoma. In 2014, further changes were made to the scoring system that reclassified certain morphological patterns from Gleason pattern 3 to Gleason pattern 4 (Epstein et al., 2016). Furthermore, it was agreed upon that instead of a scoring system ranging from 2 – 10, a more straightforward system ranging from 1 – 5 would be used. This addressed the confusion arising from situations where GS 7 would be reported without differentiating between 3 + 4 and 4 + 3, and the fact that scores below GS 6 were virtually no longer assigned. This also addressed patients' reactions to hearing GS 6 which sounds more threatening that "low grade" or "Gleason Group 1". **Table 1** details the new Gleason scoring patterns. These changes are expected to be used henceforth. In order to help with the transition in clinics, the new scoring system will be reported alongside the traditional Gleason Scores starting 2015 until it has become more widely utilized. For the purposes of this report, however, the previous version of the GS will be referenced.

The above mentioned changes to the Gleason Scoring system had profound effects on the distribution of GS 6 and GS 7 tumours, since morphologies typically classified as pattern 3 became pattern 4. A study reviewing over 3000 radical prostatectomies, needle core biopsies, and TURP specimens found that after the changes implemented by ISUP in 2005, the number

of GS 6 cancers decreased from 48% to 22% while GS 7 cancers increased from 26% to 68% (Huang et al., 2014). The revisions in 2014 included even more morphologies reclassified as pattern 4 instead of 3 and so presumably, the distribution has changed even more.

In this study, diagnostic tumours graded as GS 6 prior to 2014 were not re-graded using the new ISUP standard, which now consider glomeruloid glands and cribriforming, both regardless of morphology, as pattern 4 instead of 3 (Epstein et al., 2016). Had they been re-graded, the number of GS 7 tumours available for this study would likely be much larger since current standards for scoring would have reclassified many GS 6 cancers into GS 7. The GS 7 tumours obtained for this study were, however, re-graded using current standards to ensure that they were truly GS 7 tumours.

1.5 Tumour Stage

Tumour stage refers to the extent of prostate cancer spread at the time of diagnosis and is referenced using the tumour-node-metastasis (TNM) staging system. Approximately 75% of newly diagnosed prostate cancer patients present with localized disease (T1 or T2) (Cooperberg et al., 2004). PSA testing and tumour grade (GS) are detailed above. These three factors taken together are able to stratify patients into low, intermediate, and high risk of death from prostate cancer following surgery or radiotherapy (**Table 2**). Low risk patients can be safely followed with an active surveillance approach, though some patients opt for

prostatectomy (Klotz et al., 2010). High risk patients can receive radical radiotherapy and adjuvant hormonal therapy. Patients of intermediate risk disease are the most clinically heterogeneous group who are treated with either radical prostatectomy, radical radiotherapy, and adjuvant hormonal therapy (D'Amico et al., 2011).

1.6 Cribriforming and Intraductal Carcinoma

As mentioned above, one significant change to the Gleason grading system in 2005 was re-classifying small and large cribriform glands. Cribriform patterns have been associated with adverse outcomes in GS 7 patients after radical prostatectomy (Kweldam et al., 2015). Although intraductal carcinoma is not a listed morphology in the Gleason scoring, it is associated with high GS, advanced tumour stage, BCR, and metastasis (Kweldam et al., 2015). One study demonstrated that cribriforming and intraductal carcinoma found in diagnostic biopsies are associated with worse disease-specific survival and that including the two statuses in a predictive model resulted in better prognostic capability (Kweldam et al., 2016). With this in consideration, cribriform and intraductal carcinoma statuses were also evaluated in our study.

1.7 Prognostic Gene Signatures

Precision medicine is rapidly gaining attention in the field of healthcare, an approach whereby prevention and treatment are formulated in the context of the individual's molecular genetics or cellular composition (Collins & Varmus,

2015). One such example is the use of prognostic gene signatures in further differentiating patients based on the genetic profile of their tumour into different clinical outcome groups. For instance, a significant amount of research has been conducted on gene signatures for breast cancer (Sotiriou & Pusztai, 2009; Wirapati et al., 2008). In 2013, Prosigna's Breast Cancer Prognostic Gene Signature Assay was approved by the United States FDA, a test that measures the expression levels of 50 target genes in hormone receptor positive tumours to help predict risk of recurrence (Nielsen et al., 2014). Another prognostic and predictive gene signature in use for ER positive breast cancer is Oncotype Dx (Paik et al., 2004).

Studies focusing on developing gene signatures typically explore genes involved in key cancer biological capabilities, better known as the Hallmarks of Cancer (Hanahan & Weinberg, 2011). In the context of prostate cancer, many research groups have investigated and identified prognostic biomarkers for outcome. The phosphatase with tensin homology (PTEN) gene is a well-known and commonly studied tumour suppressor that negatively regulates the phosphatidylinositol 3-kinase (PI3K) signalling pathway (Stambolic et al., 1998). This pathway is known to drive cell proliferation and survival. Therefore, the absence of PTEN, allows for PI3K effectors such as protein kinase B (PKB, also known as AKT) to be activated and induce tumorigenesis (Cully, You, Levine & Mak, 2006). Alterations of PTEN have been observed in many different types of tumours and it is estimated to be involved in 30% of primary prostate cancers and

63% of metastatic prostate cancers (Wang & Dai, 2015). Cuzick et al. have investigated the prognostic value of PTEN loss in men with localized prostate cancer in predicting prostate cancer death and found that in a univariate analysis, PTEN loss was a significant predictor (HR: 3.51; $p<0.05$) (2013). It also proved to be a significant predictor in a multivariate analysis with GS and PSA levels; however, these results were only reflective of men with low risk prostate cancer, defined in this study as GS 6 or below, low PSA levels or low Ki-67 staining. Ding et al. investigated a gene signature consisting of four genes which demonstrated that PTEN, in conjunction with SMAD4, SPP1, and cyclin D1 were prognostic for lethal metastasis and BCR (2011). Recently, a meta-analysis of somatic copy number alterations in prostate cancer patients demonstrated an association between PTEN deletion and higher levels of overall genomic alterations (Williams, Greer & Squire, 2014). This suggests that PTEN may have a role in the genetic integrity of other regions of the genome.

TMPRSS2 and ERG are often studied concurrently since fusion occurs frequently between the two genes on chromosome 21 in prostate cancer (Tomlins, 2008). Found in approximately 50% of prostate cancer patients, the TMPRSS2:ERG fusion gene causes significant up-regulation of the ERG pathway which accelerates prostate cancer growth by increasing cell invasion and inhibiting prostate epithelial differentiation (Shah & Chinnaiyan, 2009; Yin et al., 2011). The TGF-β/SMAD3 signaling pathway plays a significant role in regulating normal and cancerous prostate cells through proliferation,

differentiation and apoptotic processes. Fang et al. were able to demonstrate that increased activity of ERG due to fusion with TMPRSS2 induced transcriptional activity of TGF-β/SMAD3 pathway by stabilizing the phosphorylated SMAD3 protein (2014). Since the TMPRSS2:ERG gene fusion is so common in prostate cancer, one study attempted to find associations between the fusion gene and clinical or pathological characteristics that may assist in predicting prognosis (Krstanoski et al., 2016). Using FISH analysis on 148 prostate cancer patients, it was found in 42% cases, but had no association with age, preoperative PSA levels, or GS, which was also reflected in other studies (Magi-Galluzzi et al., 2011; Pettersson et al., 2012); however, TMRPSS2:ERG fusion was reported to be significantly associated with stage 3 tumours. Though it cannot be conclusively said that this fusion gene can predict prognosis, it may be an asset when used in conjunction with other genes such as in a gene signature.

Research groups interested in developing gene expression signatures often analyze microarray datasets from watchful waiting studies in order to ascertain associations between patients' molecular signatures and their clinical outcomes. Markert et al. recognized that some patients with a lower risk GS such as 6 or 7 still developed aggressive disease and experienced poorer survival outcomes than expected (2011). As a result, Markert et al. examined three published prognostic gene signatures: 1) the embryonic stem cells (ESC) signature, 2) the induced pluripotent stem cells (iPSC) signatures, and 3) the polycomb repressive complex-2 (PRC2), and applied them to two independent datasets: a cohort of the SWW

(n=281) and the Memorial Sloan-Kettering cohort (n=150). They reported that tumours with a high expression of ESC genes were associated with poor survival and high GS tumours, while tumours with a high expression of PRC2 genes tended to be found in low GS tumours. When the researchers included the status of p53 and PTEN along with the above mentioned signatures, they found even more striking results. Patients undergoing watchful waiting with tumours expressing ESC genes and absence of p53 and PTEN had a 3.2-fold increased mortality risk compared to patients in other groups. This trend was also reflected in GS 6 or 7 patients, but at 2.7 times increased risk for mortality.

A discovery study attempted to examine the gene expression profiles of 6100 genes for patients in the Swedish Watchful Waiting cohort (Sboner et al., 2010). They were unable to determine a molecular model that performed significantly better than established clinical models to differentiate patients who died of cancer from patients who survived more than 10 years without metastasis. This study did report one significant association, ERG rearrangement, with lethality. Studies conducted prior to 2012 published promising results, but like many subsequent gene expression signature studies in prostate cancer, they lacked the necessary level of power or were not able to demonstrate clear superiority over established clinical factors (Penney 2011; Best 2003, Lapointe 2004; Singh 2004; True 2006).

In order for prognostic gene signatures to transition into a robust clinical assay, their prognostic capabilities must be analytically validated to provide

reproducible, accurate results, predict clinically relevant end points, and demonstrate equal performance quality for many patient types (Cullen et al., 2014). While the above mentioned studies have presented promising results that aid in our understanding of molecular genetics and tumour progression in prostate cancer, only a few have led to the development of a robust clinical assay that has been approved for clinical use. A study conducted by Klein et al. investigated a 17-gene assay to predict prostate cancer aggressiveness which involved testing the multi-gene expression signature on retrospectively collected needle biopsies of low to intermediate risk prostate tumours (Klein et al., 2014). The outcome measures of this study include clinical recurrence, prostate cancer death, and adverse pathology at prostatectomy. It was reported that the assay was able to improve prediction of BCR after radical prostatectomy as well as discrimination between high-grade and low-grade prostate cancer patients undergoing active surveillance. A similar study conducted by Cullen et al. also investigated the same 17-gene assay on its ability to associate gene expression with biochemical and metastatic recurrence for low to intermediate risk prostate cancer (Cullen et al., 2014). This study reported that the assay was able to predict time to BCR, metastasis and adverse pathology after radical prostatectomy for patients with Gleason pattern $\geq$4. As a result, a commercially available assay developed by Genomic Health, named OncotypeDX Prostate, was introduced. The OncotypeDX Prostate test is a multi-gene RT-PCR expression assay developed for formalin-fixed paraffin embedded (FFPE) prostate needle-core biopsies that measures the

expression of 17 signature genes, 12 of which are cancer-related genes associated with stromal response pathways, androgen signaling pathways, and a proliferation pathway. The remaining 5 genes are housekeeping genes. The test is available for recently diagnosed prostate cancer patients who fall under low risk or intermediate risk, meeting the criteria outlined in **Table 3**.

Two other genetic tests are available for prostate cancer patients. Myriad Genetics offers a commercially available FDA-approved prostate test called Prolaris which evaluates the expression of cell cycle progression genes. 31 cell cycle progression genes and 15 housekeeping genes are tested using qRT-PCR to produce a final cell cycle progression score. This score was found to be highly predictive for BCR and prostate cancer-specific mortality in radical prostatectomy patients and prostate cancer-specific mortality after a median follow-up time of almost 10 years in active surveillance patients (Bishoff et al., 2014). Prolaris is available to patients who are diagnosed with very low risk or low risk disease, which are typically characterized by a GS $\leq$ 6, tumour stage $\leq$ T2a, and PSA $\leq$ 10ng/mL. Decipher, developed by GenomeDx Biosciences Inc., is another genetic test available to prostate cancer patients, though it has not been FDA-approved. It examines the expression levels of 22 genes associated with cell proliferation and differentiation, cell structure, adhesion and motility, cell cycle progression, and mitotic processes, and can be performed on FFPE tissue from radical prostatectomies. It was reported that Decipher is able to predict BCR and metastasis after radical prostatectomy, but only in higher risk disease (Erho et al.,

2013). A summary of the above-mentioned information on prostate cancer genetic tests can be found in **Table 3** and **Table 4**. Clearly, there are available genetic tests for low risk and high risk prostate cancer patients, but an absence of a clinically validated, FDA-approved test specifically for intermediate risk, GS 7 patients.

RATIONALE

Our interest is in intermediate risk, GS 7 patients; that is, patients with GS 7 tumours confined to the prostate with low circulating levels of PSA (<100 ng/mL). GS 7 is the second most frequently diagnosed score in prostate cancer patients, accounting for approximately 40% of all incident prostate cancers. Patients with intermediate risk prostate cancer are a heterogeneous cohort in that it is difficult to determine their prognosis. One study found that approximately 60% of these patients are expected to have excellent long-term outcome, but the other 40% will recur despite optimal current treatment (Sboner et al., 2010). The difficulty in differentiating intermediate risk patients likely to recur from those likely to have indolent disease can lead to over-treatment and subsequently, significant patient morbidity. Much work has been conducted on furthering our understanding of prostate cancer and finding biomarkers for outcome to better stratify prostate cancer patients. Although many are in development, there is currently no prognostic gene signature or clinical assay available to stratify GS 7 patients to allow for more appropriate treatment.

BACKGROUND - PROGNOSTIC GENE SIGNATURE DISCOVERY

Our lab is interested in addressing the need for a prognostic gene signature to reduce over-treatment of prostate cancer patients. To develop this gene signature, Dr. Robin Hallet, a post-doctoral fellow in the Hassell lab, conducted the initial discovery phase experiments *in silico* to identify potential prognostic gene signatures of interest. Three gene signatures were derived from publically available gene expression profiles of the SWW cohort. This study was conducted in Örebro and South East Health Care Regions of Sweden from 1977 to 1999 and involved over 1,200 prostate cancer patients undergoing watchful waiting. Dr. Hallet's signature was based on the gene expression profiles of a small cohort of the SWW patients studied (Sboner et al., 2010). This was a cohort of 281 patients: 83 patients with GS 6 tumours, 117 patients with GS 7 tumours and 81 patients with GS 8-10 tumours. Up to 30 years of clinical follow-up information was available, all patients had prostate-localized disease at diagnosis (T1-T2) and all were followed by observation alone. The dataset can be downloaded on NCBI's gene expression omnibus using the keyword "Swedish Watchful Waiting prostate cancer progression".

In the first signature, termed the Genomic Grade Index (GGI), the top 25 genes, identified using a PAM algorithm, were associated with either low risk GS 6 (n = 83) patients and high risk GS 8 to 10 patients (n = 81). 10-fold cross validation was utilized. GS 7 tumours were not included in the identification of this signature. This signature was then tested on the GS 7 patients (n = 117) and it

was found to be significantly associated with good outcome (HR: 1.9; *p=0.0054). The results are found in **Figure 3**.

The second approach, termed the Lethal Gene Score (LGS), consisted of the top 25 genes that differentiated between indolent and lethal disease within only GS 7 patients. Patients with indolent disease (n = 22) were defined as those who were alive and well 10 years after diagnosis, while patients with lethal disease (n = 6) were defined as those who died within 2 years of diagnosis. The prognostic power of the LGS was tested on the remaining 89 GS 7 patients who were not included in the initial analysis and it was found to be significantly associated with good outcome (HR: 1.7; *p < 0.025). The results are found in **Figure 4.**

Taken together, it was found that the GGI and the LGS led to improved prognostic stratification of GS 7 tumours (HR: 2.7; *p<0.0001). As shown in the **Figure 5**, the 10 year survival of high risk GS 7 patients was approximately 30% while low risk GS 7 patients was approximately 70%. This was reflected in both GS 4 + 3 patients and GS 3 + 4 patients. The combined signature will henceforth be referred to as GGI+LGS.

An additional analysis was conducted on the GS 7 patients of the SWW cohort using a network-based approach. Network-based medicine is a concept introduced by Barabasi and Albert that applies the understanding of complex interactions in diverse systems, such as computers, to the field of medicine and

genetics. In his publication in 1999, he noted contemporary science's difficulties in describing systems composed of enormous amounts of proteins and genes and the chemical interactions between them (Barabasi & Albert, 1999). He proposed that despite having a wealth of knowledge about individual cellular components, the key to understanding diseases and functional abnormalities lies in understanding the complex interactions between proteins, nucleic acids, and other small molecules. Furthermore, he proposed that the manifestation of disease phenotypes were unlikely the result of abnormalities found in single genes and more likely a result of the malfunction of biological components interacting with each other in a complex network. In addition, by using a network-based approach that accounts for the interactions of gene products, the potential for noise and false positives is decreased (Barabasi, Gulbahce, & Loscalzo, 2010). As a result, Dr. Hallet used a network-based approach to determine additional genes that might be prognostic in a cohort of GS 7 SWW patients (n = 177), utilizing tumour scores obtained using Illumina K6 DASL gene expression technology. To identify prognostic genes, a univariate cox-regression analysis was conducted on each oligonucleotide probe comprising the 6K gene panel. 366 probes were identified that were significantly associated with outcome in the GS 7 cohort of patients ($p<0.05$), and subsequently mapped as nodes onto the human functional protein interaction network (Reactome). 88 genes were found to be significantly associated with outcome, consisting of 10 distinct network modules (Wu, Feng, & Stein, 2010). Each module contained between 7 to 10 genes which corresponded

to a specific biological pathway. A univariate cox-regression analysis on each separate module demonstrated that each one was significantly associated with patient outcome (**Table 5**). Taken together, it was found that the mean of the 10 individual module scores demonstrated even better prognostic capabilities than each individual module. Using these 88 genes, it was found that GS 7 patients were accurately stratified into high risk and low risk groups resembling the survival curves of low risk GS 6 and high risk GS 8 patients respectively (**Figure 6**). The prognostic capacity of the network gene signature was then tested *in silico* on patients of the Mayo Clinic cohort, a study which also examined the gene expression profiles of 545 patients with early stage prostate cancer with a median follow-up time of 16.9 years (Erho et al., 2013). Similar results were obtained in that GS 7 patients were accurately stratified into a group at high risk for poor outcome and a group at low risk for poor outcome. Those patients deemed high risk GS 7 by the signature had outcomes that resemble patients with GS 8 tumours while those patients identified by the signature as low risk had outcomes that resembled those patients with GS 6 tumours.

With these encouraging results, we hope to validate the strength of our signature genes in the FFPE tumour tissue of prostate cancer patients. For both the GGI and the LGS signatures, 24 of the 25 genes were ultimately selected for NanoString analysis; code sets were unable to be made for one gene from each signature. Although there were no common genes between the GGI and the LGS, both signatures contained 4 genes that were also found in the Network signature. 3

additional genes were also included in the analysis: PTEN, TMPRSS2, and ERG, and 6 housekeeping genes were used. In total, our prognostic gene signature consists of 141 genes.

OBJECTIVES

The *in silico* discovery phase has identified 3 gene signatures that have demonstrated an ability to divide intermediate risk prostate cancer patient samples into more prognostically relevant subgroups. By improving prognosis, treatment plans that are better suited for individual patients can be pursued. Promising assays that are commercially available have been recently developed to improve stratification for low-risk GS prostate cancer; however, no such assay exists for patients with intermediate risk, GS 7 tumours.

The objective of this work is to prospectively validate our prognostic gene signatures, discovered by Dr. Hallet, which collectively consist of 141 genes using a retrospective cohort of prostate cancer patients on whom long term follow-up data is available. The 141 genes will be tested on intermediate risk, GS 7 prostate cancer patients enrolled in the National Cancer Institute of Canadian Clinical Trials Group (NCIC-CTG) PR5 randomized trial, with the expectation that it will be able to stratify this patient population into a group at high risk and a group at low risk for biochemical and metastatic recurrence. These objectives will be met by:

1. Collecting FFPE tumour samples from various Ontario heath care centres

2. Rescoring samples' H&E slides to confirm GS and obtain additional pathological information (presence of cribriforming, intraductal carcinoma, etc.)
3. Perform RNA extraction
4. Perform NanoString nCounter analysis on previously identified (n=141) genes
5. Test prognostic capacity of gene signatures against each other and against clinical information obtained from tumour samples and clinical trial notes

METHODS

5.1 The PR5 Trial and Accrual of FFPE Samples

The PR5 trial was a prospective randomized trial conducted between 1995 and 1998 in 8 Ontario regional cancer centres and 8 additional Canadian centres, coordinated by the Ontario Clinical Oncology Group and the NCIC-CTG (Lukka et al., 2005). It investigated two different fractionation schedules for patients with localized prostate cancer. A total of 936 men with early stage (T1 or T2) prostatic adenocarcinoma were randomized into either 66 Gy in 33 fractions over 45 days or 52.5 Gy in 20 fractions over 28 days. Clinical follow-up information on all patients was collected until 2008, resulting in a median follow-up of over 12 years.

The primary outcome of this trial was biochemical failure which was defined as any one of the following events: PSA failure as outlined by the Houston definition, clinical evidence of metastasis (local and/or distant), commencement of hormonal therapy, or death due to prostate cancer. Low, medium, and high risk GS patients were included in this study ranging between GS 2 to GS 9. Patients were excluded if they presented the following: a PSA level of more than 40 ng/ml, previous therapy for prostate carcinoma (other than biopsy), or prior or active malignancy not including non-melanoma skin cancer, colon cancer, or thyroid cancer treated at least 5 years before the trial and presumed cured. After treatment, patients were examined at 4 weeks, 6 months,

and every 6 months thereafter. Each visit included an update on medical history and physical examination with a digital rectal exam (DRE). PSA levels were measured starting at the first 6-month post-treatment examination. FFPE blocks were obtained from the diagnostic core biopsies from each patient.

260 patients with GS 7 intermediate risk prostate cancer from the PR5 trial were chosen for this study. These patients' original diagnostic prostate core biopsy samples were housed in their original pathology departments of their host institutions and not centrally collected as part of the trial protocol; thus, a significant amount of time for the purposes of this study was dedicated to collection of these tissue samples from approximately 30 health care institutions across Ontario. The REB at Hamilton Health Sciences approved the collection of FFPE prostate biopsy blocks from the PR5 trial. In addition, 15 health care centres requested submission of a separate application specific to their REB.

5.2 Rescoring H&E slides

An H&E stained slide was made from each FFPE block and scored by the study pathologist, Dr. Anita Bane. The following information was obtained from each slide: GS (if GS 7, whether it was GS 3+4 or 4+3), type of biopsy (needle-core biopsy or TURP), number of cores containing tumour, length (mm) of tumour tissue involved per core, percentage of biopsy area containing tumour, presence of cribriforming, presence of intraductal carcinoma, and whether cores displayed discontinuation greater than 5 mm. Scoring was done using the updated

2005 ISUP Gleason Grading System. It should be noted that some health care centres did not allow the release of FFPE blocks, but agreed to provide H&E slides and sections. A copy of our sectioning protocol was provided to those centres to follow.

5.3 RNA Extraction

Four different RNA extraction kits from Roche, Qiagen, Promega, and NanoString were compared for their ability to extract the highest amount of RNA from test samples of prostate and breast FFPE tissue. Based on the results shown in **Figure 9**, Promega's ReliaPrep™ kit was chosen for this study. As previously mentioned, the top slide was stained with H&E and scored by the study pathologist. During this scoring step, tumour areas were measured and marked. In most cases, 10 sections at 5 μm thickness were obtained from each prostate FFPE block for RNA extraction. In others, specifically blocks containing less than 3 mm in length of tumour, 12 sections of similar thickness were obtained to account for anticipated low levels of extracted RNA.

RNA extraction was conducted in an RNAse-free environment. Tumour tissue was manually dissected from unstained slides using an RNase-free razor blade and placed in a microcentrifuge tube. Typically, RNA extraction protocols for FFPE tissue include a primary step in which paraffin is dissolved from the tissue. In accordance with Promega's ReliaPrep™ kit, the majority of the excess paraffin was manually removed, but trace amounts were added along with tumour

tissue into the microcentrifuge tube, which was filtered out in later steps. RNA concentrations were determined using BioDrop. The majority of samples were normalized to 13 μL containing 250 ng of RNA for NanoString analysis. In some cases, it was not possible to meet the 250 ng of RNA even when additional sections were included; however these samples of lower RNA yield fell within the recommended minimum range of volume for successful NanoString analysis (between 150-200 ng). A260/280 levels were also determined using BioDrop. RNA A260/280 values greater than 1.8 are considered to be an indication of high RNA purity (Boeckx et al., 2011; Fleige & Pfaffl, 2006). A small number of samples were analyzed using the Bioanalyzer to examine RIN values and nucleotide length.

5.4 NanoString Analysis

Moved to discussion section ----> For every gene target of interest, two 50 base sequences are made that are complementary to a 100 base target region. The first 50 base pairs are covalently linked to a biotin molecule and is called the capture probe. The other pair is covalently linked to a colour-coded molecular bar code and is called the reporter probe. A solution-based hybridization is formed in the presence of excess probe in order to pair up the two probes with the target of interest. Up to 800 probe pairs can be formed in a single reaction. The hybridized complexes are purified via an automated liquid handling robot and bound to a streptavidin surface for imaging. The hybridized complexes are elongated and immobilized in order to be read by the digital analyzer. The scanner then

individually counts the number of bar codes hybridized to a single RNA or DNA molecule. Since there is no amplification process, a single molecule is equal to a single count. This allows for a high degree of precision and sensitivity over a large dynamic range. A final sample size of 75 was obtained, consisting of samples that were not flagged for content normalization.

5.5 Test Prognostic Gene Signatures

The primary interest of this project is the prognostic capacity of the GGI+LGS and the Network signature to predict BCR or metastatic event. Specifically, it would be interesting to know which of the two signatures are superior. In addition, which of the 2 components that makes up the GGI+LGS is superior and which of the 10 components that make up the Network signature is superior. A univariate cox regression analysis will be conducted to determine any association between gene expression and hazard for BCR or metastatic event.

The secondary interest of this project is whether morphological information obtained from scoring H&E slides is superior for predicting BCR or metastatic event. A univariate cox regression analysis will be conducted to determine any association between tumour length and poor outcome. A chi-squared test of independence will be conducted to determine whether GS or the presence of cribriforming or intraductal carcinoma is associated with poor outcome. Furthermore, it would be interesting to know whether the addition of well-studied prostate cancer genes such as PTEN, TMPRSS, and ERG could

enhance the prognostic capacity of our established gene signatures, since many studies have alluded to their importance in prostate cancer development. Although these three genes were not included in the initial *in silico* discovery phase, they were added to the NanoString nCounter analysis and are included in the total 141 tested genes. A univariate cox regression analysis will be conducted to explore the above. A summary of the primary and secondary questions can be found on the following page.

Primary Questions

1. Is the GGI+LGS signature able to accurately predict patients at a high risk for BCR or metastatic event?
2. Is the Network signature able to accurately predict patients at a high risk for BCR or metastatic event?
3. Which of the two, between the GGI and the LGS, is able to accurately predict patients at a high risk for BCR or metastatic event?
4. Which of the 10 individual modules that make up the Network signature is able to accurately predict patients at a high risk for BCR or metastatic event.

Secondary Questions

1. Is the predictive capacity of the GGI+LGS improved when combined with the Network signature?
2. Is the predictive capacity of the GGI+LGS or Network signature improved when combined with PTEN, TMPRSS, and ERG?
3. Is the predictive capacity improved if both signatures together are combined along with PTEN, TMPRSS, and ERG?
4. Is GS superior to our gene signatures when predicting BCR or metastatic event?
5. Is the presence of cribriforming and intraductal carcinoma associated with patients at a higher risk of BCR or metastasis?

6. Is the total length of tumour measured on a needle core biopsy associated with outcome?

RESULTS

6.1 Accrual and Data Collection

27 Ontario healthcare centres were contacted, 15 of which required re-approval from their respective REB's. Clinical follow-up information was collected from each case which included date of birth, date of first consultation, date of BCR, date of metastasis, and date of death. Due to technical difficulties, not all healthcare centres were able to locate or deliver the blocks required. It was conservatively estimated that we would amass a total of 60% (n = 156) of the 260 GS 7 patients enrolled in the PR5 trial. A final total of 132 FFPE blocks were recovered from the associated Ontario health care centres.

12 patient blocks were found to have an ineligible GS, either less than or greater than a GS of 7. 9 cores did not contain any viable prostate tissue for analysis. 27 samples did not contain adequate amounts of RNA within the range of 150-200 ng required for NanoString analysis. 9 samples were flagged by NanoString's nCounter software for failing to meet normalization criteria. A final total of 75 samples containing enough GS 7 tumour tissue for RNA extraction and analysis through NanoString were obtained. Accrual results are illustrated in **Figure 8**.

Each H&E slide was scored by the study pathologist. Regions of tumour were circled for RNA extraction and additional pathological characteristics were recorded. Examples of scored slides are shown in **Figure 12.** 3 of the 75 samples

were TURP biopsies instead of needle-core, but were nonetheless included for RNA extraction and subsequent analysis. Tumour length ranged from 1 mm to 48 mm. Samples containing much smaller amounts of tumour compared to others, such as those with 3 mm or less, were given two additional sections when possible to compensate for expected lower RNA extraction values. This was not done if the additional sections would exhaust all tissue in the FFPE block. Full results of rescoring are outlined in **Table 3**. 24 cases of cribriforming and 4 cases of intraductal carcinoma were found. Examples of each morphology are illustrated in **Figure 13** and **Figure 14**. Gleason Grade 3 and Gleason Grade 4 morphologies are illustrated in **Figure 15**.

RNA extraction was completed using the Promega ReliaPrep^{TM} kit. Quantity and quality of RNA was examined using BioDrop. The average concentration of RNA extracted was 38.67 ng/µL at a standard deviation of 28.43 ng/µL. The integrity of the RNA, established using the A260/280 value, was an average of 1.85 with a standard deviation of 0.06. Full results of the extraction are outlined in **Table 7**. 12 test samples were analyzed using the Bioanalyzer and results can be found in **Figure 11**. As expected, RIN values were found to be extremely low; however, high RIN values are not required for a successful NanoString analysis. The average percentage of RNA molecules greater than 300 base pairs was 22.5%. Despite these suboptimal values, NanoString analysis can proceed because concentration values were met, at a reasonably and consistently high purity level. Samples were stored in –80 degrees Celsius and transported to

the Farncombe Metagenomics Facility at McMaster University for NanoString analysis.

6.2 Data Analysis – Normalization

NanoString results are viewed on NanoString's nCounter software. nCounter's first normalization step involves normalizing all gene expression values to an External RNA Control Consortium positive control in order to assess technical performance. Typically, 6 different positive controls are spiked into the experiment. It is expected to see decreasing counts starting from Positive Control A to Positive Control F. Negative controls should contain uniformly low counts, as observed. A table of the data at a $\log_{10}$ based scale is shown below in **Figure 10** which reflects the obtained results. The geometric means of the positive control values were calculated for each sample which was then used to calculate a normalization factor. This factor was then applied to each gene expression value obtained in the experiment, including housekeeping genes.

The second step involves normalizing all gene expression values to the housekeeping genes which involved the same mathematical process of taking the geometric mean of each sample's expression values to calculate a normalization factor. This factor was then applied to each gene expression value obtained in the experiment. The expectation is that housekeeping genes will be uniformly expressed in all tissue types since these genes are responsible for basic cell maintenance. Recent studies conducted on the utility of housekeeping genes,

particularly a common one such as GAPDH, have found variability of their expression levels which puts into question their use in gene expression analysis (Eisenberg & Levanon, 2013); however, this variability was observed across different types of tissue, not within the same type. It is recommended that combinations of genes are used as a reference (Sharan, Vaiphei, Nongrum, Keppen, & Ksoo, 2015). It is acceptable that in this study, our housekeeping genes are used because the only tissue type in question is prostate tissue. Expression values were graphed and can be found in **Figure 16** which demonstrates that there was uniform expression of housekeeping genes across all 75 samples. Once normalized gene expression values were obtained, all data was then log2-transformed in order to decrease the range of expression levels which expanded over several orders of magnitude (Kreil & Russell, 2005).

6.3 Data Analysis

Data was analyzed using the SPSS Statistics software, PASW Statistic 18. A univariate cox regression analysis was conducted to explore the utility of our gene signatures in predicting BCR or metastatic event. Significance was established at p-values < 0.05. Statistical results of the analysis for primary and secondary questions can be found in **Table 8** and **Table 9**.

An examination of the primary questions showed that when taken separately, the expression values of the GGI+LGS signature were significantly associated with outcome (HR: 0.100; *p=0.002; 95%CI: 0.024-0.427) while the expression values of the Network signature were not (HR: 0.306; p=0.155;

95%CI: 0.060-1.563). Each signature was subsequently broken down into its components and analyzed. From the GGI+LGS, a significant prognostic association was found between the GGI signature and outcome (HR: 0.178; *p=0.000423; 95%CI: 0.068-0.465) suggesting that each unit increase of GGI expression is associated with an 82.2% decrease in hazard for poor outcome. When the Network signature was separated into its 10 modules, 3 modules were found to be statistically significant for their ability to predict outcome. M1, consisting of epigenetic genes, was associated with 49.4% decreased hazard for poor outcome when highly expressed (HR: 0.506; *p=0.006; 95%CI: 0.311-0.824). M3, consisting of genes related to TGF-β, integrin, growth factor receptor signaling, was associated with a 45.4% decrease in hazard for poor outcome when highly expressed (HR: 0.546; *p=0.006; 95%CI: 0.354-0.844). M7, consisting of genes related to epigenetics, was associated with a 72.2% increase in hazard for poor outcome when highly expressed (HR: 1.722; *p=0.013; 95%CI: 1.124-2.638).

Secondary questions addressed the utility of our gene signatures if signatures were combined or altered and if clinical factors were superior to gene signatures for prognosis. A combined signature of both the GGI+LGS and the Network signature yielded a statistically more significant result compared to when they are taken separately (HR: 0.022; *p=0.002; 95%CI: 0.002-0.216). The addition of well-studied prostate cancer genes such as PTEN, TMPRSS, and ERG did not improve the utility of either gene signatures, taken separately (Network –

HR: 0.821; p=0.220; 95%CI: 0.599-1.126) (GGI+LGS – HR: 0.808; p=0.179; 95%CI: 0.592-1.103) or together (HR: 0.762; p=0.162; 95%CI: 0.520-1.115). Clinical factors were also examined for their prognostic capacity. GS, either 3 + 4 or 4 + 3, was not found to be significantly associated with outcome (HR: 1.090; p=0.806; 95%CI: 0.546-2.177). Total tumour length was found to be associated with a greater risk for poorer outcome (HR: 1.044; *p=0.020; 95%CI: 1.007-1.082). A chi-squared test for independence was conducted to examine whether the presence of cribriforming and intraductal carcinoma is associated with a higher hazard for outcome. No significant association was found for cribriforming (χ^2=0.874; p=0.457) or intraductal carcinoma (χ^2=0.705; p=0.622). **Figure 18 – 22** outline the average expression levels of all signature genes, categorized by their respective signatures.

DISCUSSION

This study was designed to investigate the capacity of our pre-established prognostic gene signatures for predicting BCR or metastatic event in GS 7 prostate cancer patients using their archival, diagnostic FFPE tissue. A total of 141 genes were analyzed using NanoString technology. Pathologic tumour variables were also explored for their prognostic capacity. **Table 10** outlines all genes involved and **Table 6** outlines the morphological results from slide review and GS.

The principal finding of this study was that the gene expression levels of the GGI+LGS were associated with BCR or metastatic event (HR: 0.100; *p = 0.002). Each unit increase of the average expression of GGI+LGS genes led to a 90% decrease in risk for poor outcome. The GGI signature (HR: 0.178; *p = 0.000423) had a greater contribution to the GGI+LGS's predictive capacity than the LGS signature (HR: 0.595; p = 0.362), demonstrating that for every unit increase of average expression of GGI genes, there was an 82.2% decrease in risk for poor outcome. As shown in **Figure 18**, the top two most highly expressed genes were PTTG1 and DYNC1I1.

PTTG1 overexpression is typically associated with endocrine-related tumour formation and metastasis, though its downstream effects are still being investigated (Lin et al., 2015; Castilla et al., 2014). In a normal cell, expression of PTTG1 is only up-regulated during an active cell cycle, which suggests its

involvement in cellular proliferation. The exact molecular mechanisms of how PTTG1 promotes proliferation are still not fully understood although there is some evidence that PTTG1 is linked to TGF-β signalling pathways through downstream regulation of SMAD3 (Huang et al., 2014). Many studies have observed its oncogenic effects when overexpressed; however it is also known to be involved in DNA damage repair, apoptosis, and angiogenesis (Castilla et al., 2014). In fact, PTTG1's overexpression has been linked to both promotion and inhibition of apoptosis (Vlotides, Eigler & Melmed 2007). Based on our results, it seems that PTTG1 overexpression contributes to an overall protective effect in our cohort of patients, which is contrary to evidence suggesting that it is a key player in cancer growth models. One study found that high PTTG1 protein levels were associated with higher apoptotic induction in a prostate cell line, but only after paclitaxel treatment (Castilla et al., 2014). It is reasonable to hypothesize that the consequences of high PTTG1 protein levels are dependent on additional factors, such as the progression of mitosis. When it is stopped due to the presence of mitosis-interrupting treatments, high PTTG1 levels may then induce the cell to undergo apoptosis rather than cellular growth when mitotic function is normal. This ideal environment where one is able to observe PTTG1's protective effects may well be found in the tumours of the PR5 patients who were treated with radiation. Cellular growth can be halted when cancer cells are radiated, either through the production of free radicals which damage DNA or through direct

DNA damage. This would provide the right conditions for PTTG1 to illicit its apoptotic effects.

DYNC1I1 is one of several protein coding genes that are involved in the linkage of a motor protein, cytoplasmic dynein, to other intracellular components. Together, they contribute to intracellular transportation via cellular microtubules and organelle positioning. Cytoplasmic dynein also plays an essential role during cell division including centrosome separation and spindle positioning (Raaijmakers & Medema, 2014). Although the current literature lacks studies conducted directly on cytoplasmic dynein's role in prostate cancer development and progression, one study has suggested that spindle and nuclear movement are impaired in cancer cells (Bierle et al., 2015). Another interesting study on cytoplasmic dynein 1 examined its mechanism of action on a known anti-tumour molecule, Amblyomin-X, a protein isolated from the salivary glands of a tick. Amblyomin-X is able to inhibit proteasomes, leading to pro-apoptotic effects and decreased tumour growth. The study found that cytoplasmic dynein was a significant contributor to Amblyomin-X uptake into human tumour cell lines and delivery to its primary target, the proteasome (Pacheco et al., 2015). These studies suggest that DYNC1I1 may play an important role in maintaining the integrity of intracellular and nuclear dynamics, which may allow for normal processes such as proper mitosis and apoptosis to occur. Furthermore, maintaining such dynamics may be conducive to the delivery of important cancer treatments, perhaps ones that resemble the chemical structure of Amblyomin-X.

It is important to note that the success of a prognostic gene signature is dependent on the sum of all signature genes rather than the individual genes themselves. Therefore, it may be more meaningful to examine the overall pathways associated with the GGI signature, rather than individual genes, and compare them with existing pathways of prognostic gene signatures that are commercially available. Cell cycle progression, immune signalling, cellular organization, and proliferation pathways encompass majority of the genes listed in the GGI signature which happens to closely align with the pathways of interest for assays such as Oncotype Dx, Prolaris, and Decipher (**Table 12**). Although this is not conclusive evidence, it nonetheless suggests that the GGI signature may be heading in the right direction, towards the goal of becoming a commercially available prognostic gene signature for prostate cancer patients.

There was no association between the expression values of the Network signature and outcome (HR: 0.306; p = 0.155; 95% CI: 0.060-1.563). It was surprising that the network signature as a whole was unable to predict outcome, since our preliminary *in silico* analysis on the Mayo Cohort, as described on page 16, was able to demonstrate a high degree of utility. A closer examination of each individual module was conducted and subsequently, only 3 out of 10 modules were found to be predictive of outcome: M1, M3, and M7. M1 and M7 consist of genes involved in epigenetic processes, although each had opposite effects on risk for outcome. High expression of M1 genes was associated with a decrease in risk for poor outcome, but high expression of M7 genes was associated with an

increase in risk for poor outcome. This was inconsistent with the *in silico* results which suggested high expression of all modules was associated with decreased risk. M3, consisting of genes largely involved in TGF-β, integrin, growth factor receptor signaling, was associated with a decrease in risk for poor outcome when highly expressed. A likely reason for M7's observed results might be the fact that the *in silico* analysis used metastasis or cancer-specific death as an outcome rather than metastasis or BCR. Unfortunately, metastatic events in this cohort of patients were particularly low, at 6.7% (n = 5) and the causes of death were not available. BCR, although an important prognostic tool, may not be the best surrogate endpoint for cancer-specific death or even metastasis. Despite these observations, the Network signature may very well be a useful one, but only when attempting to predict certain endpoints. Although the Network signature modules are not meant to be examined in isolation, it was interesting to observe that two modules specifically relating to epigenetic processes were statistically significant in predicting outcome. Epigenetic aberrations are important cellular events, particularly in cancer biology, by which heritable external or environmental factors such as DNA methylation, chromatin remodeling and microRNAs, alter phenotypic expression without altering the genome. In the context of prostate cancer, epigenetic events may play a significant role in disease initiation and progression; global hypomethylation or low levels of 5-methylcytosine, has been found in advanced metastatic prostate tumours and hypothesized to contribute to cancer via activation of proto-oncogenes and disruption of chromosomal stability

(Chiam, Ricciardelli, & Bianco-Miotto, 2014). More recently, a signature investigating 52 differentially methylated sites found evidence supporting its predictive capacity, suggesting its applicability to stratifying GS 7 patients into better outcome groups (Geybels et al., 2016). Other therapies focusing on disrupting epigenetic-related enzymes and proteins have been observed to slow cancer cell growth are now being investigated in clinical trials (Graça et al, 2016).

The combined utility of the GGI+LGS and the Network signature (HR: 0.022; *p = 0.001; 95%CI: 0.002-0.216) seemed to be superior to each signature when tested alone, although it was only marginally better than when GGI+LGS was analyzed alone (HR: 0.100; *p = 0.002). Surprisingly, the inclusion of PTEN, TMPRSS2, and ERG lead to no significant associations with outcome. PTEN deletions and TMPRSS2:ERG fusions are commonly found in prostate cancer tumours and are well-known for being associated with oncogenic processes. PTEN deletions, although uncommon in early stage prostate cancer (< GS 7), has been reported to predict development of metastasis and prostate cancer specific mortality (Mithal et al., 2014). Perhaps one reason why this association was not observed may be the fact that PTEN expression in this cohort of patients was relatively high in comparison. The average expression level of all 141 genes was 7.76 ± 2.43 units while the average expression of PTEN was 9.79 ± 2.13, which may even be evidence that PTEN was up-regulated. Given the expression values, it is likely that the PTEN gene was active. Even if its expression values had been closer to zero, it still would have been necessary to investigate whether the PTEN

gene was either truly deleted or if its expression was simply suppressed. Furthermore, 73.3% of our GS 7 cohort was lower grade (3 + 4) which may decrease the number of potential PTEN deletions since they are typically associated with high grade prostate tumours.

TMPRSS2:ERG rearrangements and their association with prognosis is more controversial since its expression has been linked to both low grade and high grade tumours; however, more recent studies have suggested that expression levels of TMPRSS2-ERG and ERG are more common in aggressive tumours (Hernández et al., 2016). There are a few possibilities to explain our results. Firstly, the expression level of TMPRSS2 in isolation is not likely to be associated with outcome since its primary function, when rearranged with the ERG gene, is to act as a promoter (Tomlins et al., 2008). TMPRSS2 is normally highly expressed in prostate epithelial tissue, though in our cohort, its expression levels (6.96 ± 1.34) seem to be on the lower end, relative to housekeeping genes and signature genes. ERG expression was relatively higher (10.85 ± 0.96), though this is insufficient evidence that this is a result of a fusion with TMPRSS2. To directly confirm the presence of fusion, one would be required to perform 5' RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) analysis and sequencing of the product using RT-PCR (Demichelis et al., 2007).

It was interesting to observe that our gene signatures appeared to be superior to GS in predicting outcome, a finding reflected in other studies on prognostic gene signatures in prostate cancer (Erho et al., 2013). Although the GS

has maintained its reputation as a powerful prognostic marker in the clinical setting, these findings suggest that supplementing it with gene expression analysis can be a significant improvement on predictive capacity.

The presence of cribriforming and intraductal carcinoma was also recorded. As per the 2005 changes to the Gleason Grading System, small and large cribriform, ill-defined glands, and glomeruloid glands were redefined as GS 4. Cribriform growths have recently been shown in case-control study on GS 7 prostate cancer patients to be strong predictors for BCR, distant metastasis, and disease-specific death (Kweldam et al., 2015). The same study also noted the presence of intraductal carcinoma and its association with distant metastasis and BCR; however, intraductal carcinoma is associated with high-grade cancer and so, low counts are expected in this study (Van der Kwast et al., 2012). A chi-squared test of independence showed no significant association between the two morphologies and outcome.

The total length of tumour was found to be significantly associated with outcome (HR: 1.044; $p = 0.020$; 95% CI: 1.007-1.082), though the association was weak. Per unit increase of the average total length of tumour was linked to a 4.4% increase in risk for outcome. These results are not so surprising, since intuitively, greater tumour mass is expected to be a sign of more advanced disease and hence, poorer outcome. One research group investigated the prognostic value of tumour volume in radical prostatectomy specimens and reported that total tumour and index tumour volumes were significant predictors of BCR (Shin et al.,

2016). Due to the nature of needle-core biopsies, it is reasonable to question whether the length of tumour in this biopsy method is an accurate reflection of total tumour volume. Sometimes, parts of the tumour may be missed or unreachable. However, one study comparing greatest tumour length, greatest tumour percentage, and total tumour length reported that total tumour length in needle-core biopsies is indeed the best way of measuring tumour extent for small-volume prostate cancers (Kajikawa et al., 2016).

Another important finding of our study is the success of gene expression analysis using archival FFPE needle-core biopsies. FFPE is a valuable resource in the field of oncology and NanoString's compatibility with FFPE samples is one of the reasons why it is an ideal method to reliably quantify mRNA molecules from a small amount of RNA. Other types of analysis are impeded by difficulties that NanoString is able to bypass. Such examples include low yield of extracted nucleic acids and the nucleic acid degradation resulting from formalin fixation. It is well known that the process of embedding tissue into formalin blocks leads to the degradation of RNA which makes gene expression studies difficult. For instance, oxygen and hydroxyl radicals in formalin can lead to crosslinks between nucleic acid molecules, as well as high temperatures of the wax during the embedding process, can both lead to unwanted changes to RNA which then impairs molecular analysis (Knudson et al., 2016). We were able to circumvent those anticipated barriers with the use of NanoString technology, which has consistently proven its ability to provide accurate and reliable quantification of

RNA from FFPE blocks. In addition, NanoString nCounter technology is ideal for RNA extracted from FFPE material because it does not require amplification, it targets regions as small as 100 bases, and can measure up to 800 genes with small amounts of sample RNA (100 ng). The technology is simple and hands-off, reducing the chances of laboratory errors. Numerous studies have been published detailing the reliability of utilizing NanoString nCounter technology on FFPE samples (Tyekucheva et al., 2015; Veldman-Jones et al., 2014; Northcott et al., 2012; Kulkarni, 2011).

NanoString published an article in Nature Biotechnology in 2008 comparing the nCounter gene expression system against the Affymetrix GeneChip and TaqMan PCR on assaying over 500 gene expression levels. Results showed that NanoString technology was, in terms of detection sensitivity and accuracy of mRNA, superior to Affymetrix and equal to TaqMan PCR (Geiss et al., 2008). Subsequently, many other researchers began testing this method against standard ones for quantifying gene expression and found that NanoString was, at the very least, able to deliver equally satisfactory results (Veldman-Jones et al., 2014; Kolbert et al., 2013; Adam et al., 2016). These advantages are largely based on NanoString's ability to measure RNA directly without any enzymatic amplification steps which are normally required in RT-PCR. In the absence of an amplification step, gene-specific or 3' biases are no longer a concern which allow for the calculation of RNA expression using individual molecules (Geiss et al., 2008). Moreover, RT-PCR calculates expression from the number of enzymatic

reactions which makes it vulnerable to inaccurate results even in cases of minimal contamination. Lastly, NanoString is much more time and resource efficient which likely contributes to a decrease in variability. This study adds to the understanding that advancements in technology can and will improve our ability to tap into the potential of FFPE tissue for gene expression analysis.

LIMITATIONS

The lack of power due to small sample size was a clear limitation in this study. The estimated sample size of 160 could not be met due to various logistical circumstances, including but not limited to inaccessible archived FFPE blocks, incorrectly scored tissue, and a lack of viable tissue in obtained blocks. Due to recent changes to the Gleason Scoring System in 2014 that reclassified some tumour morphologies characteristic of GS 3 to GS 4, it might have been worthwhile to rescore all the GS 6 patients from the PR5 cohort since it is expected that a number of them would now be considered GS 7. This alternative, however, would not be possible due to limited resources.

With regards to the inaccessibility of archival tissue, REB applications were required for almost every healthcare facility in possession of archival tissue blocks, a process that proved to be time intensive and costly. Each REB had different procedures and regulations which made time management difficult, seeing as some REB's requiring an inordinate amount of time to deliver samples. A centralized REB for the province of Ontario dedicated to retrospective tissue studies would have been a significant improvement, especially considering how common FFPE studies are.

There were also some limitations regarding the accuracy of the final sum of GS 7 samples, not pertaining to the 2014 Gleason Scoring changes. Firstly, although needle core biopsies are the current standard in prostate diagnosis, this

procedure is not able to address the heterogeneity and multifocal nature of prostate tumours which may ultimately mask the true GS of a tumour (Knudsen et al., 2016; Boutros et al., 2015). Secondly, reproducibility can be difficult between pathologists when utilizing the GS which may have also altered the final sample size (Chang, Autio, Roach, & Scher, 2014). Overall, these factors may have reduced the power of this study, though most likely to a small degree.

Another limitation of this study centres on the specific use of the SWW microarray data to generate our prognostic gene signatures. Perhaps the signatures were inherently overfit for the specific population involved in the SWW cohort and was unable to perform as well in other types of population such those patients living in Canada involved in the PR5 trial. This highlights the importance of re-testing in another cohort of patients to ascertain whether our signatures can be applied to a broader scope of patients. Furthermore, the prostate tumours of the SWW cohort were scored using a much outdated Gleason Scoring criteria and likely affected the prognostic strength of our signatures. If possible, the SWW cohort should be reclassified into their appropriate GS group according to current GS standards. RNA is particularly sensitive to external factors such as freeze-thaw events which had to occur during transportation between labs and so, final gene expression values obtained may be imprecise. Specific to freeze-thaw events, this limitation can be addressed by using dry ice and using a more time-efficient method of transportation to reduce the degree of thawing. Interestingly, one study compared different degrees of automation for RNA extraction from archival FFPE

tissue and found that a fully automated method led to the best reproducibility in gene expression analysis (Bohmann et al., 2009). This level of automation likely eliminated the possibility of technical errors and reduced the presence of contaminants which may cause unwanted alterations in the RNA extraction process. These improvements parallel those which have been demonstrated through NanoString technology, which has essentially automated and simplified conventional gene expression assays. If resources were available to allow for a completely hand-off approach to RNA extraction, we may have observed more accurate results.

Understandably, resources are often scarce and improvements that are resource intensive may not be possible; however, it is imperative that the pre-analytical phase of the total testing process is rigorously established in order to reduce errors, particularly considering that most errors originate in pre-analytics (Da Rin, 2009). Developing easy-to-follow procedures, providing training, improving information technology, and introducing automation are just some examples of reducing sample collection and handling errors. This project touched on several pre-analytical factors such as the ideal environment to obtain an ideal number and thickness of tissue sections required for RNA extraction. Factors that were not discussed and perhaps were out of our control include details regarding the prostate biopsy and the method of fixing the tissue in blocks. When transitioning into a commercially available assay, the pre-analytic phase should be

clearly outlined so as to reduce total testing process errors and enable analysis of high quality and standardized samples.

Although NanoString nCounter has proven its ability to address limitations that would have been present in other gene expression techniques, there are some setbacks that are noteworthy regarding accuracy and normalization. Each NanoString experiment quantifies 10 samples at a time, meaning that a study including over 100 samples would require over 10 submissions (10 samples each). Variations within each submission were not accounted for and an investigation into any differences that might exist between submissions would be important for knowing whether the final gene expression data is reliable.

CONCLUSIONS AND FUTURE DIRECTIONS

Our study has shown that prognostic gene signatures can improve on our current methods of predicting prostate cancer patient outcome by adding crucial information about patients' tumours' genetic composition. Although the results in our prognostic gene signature discovery phase were not exactly reflected in this study, several subcomponents of our signatures were able to predict risk for outcome at a statistically significant level. High levels of gene expression associated with intracellular protein transport, apoptosis, immune processing, and co-chaperones, were observed to be associated with a decrease in risk for BCR or metastasis. In fact, it was shown that these gene signatures were even better than established predictive indicators such as GS or cell morphology; however, the low statistical power of this study should be noted along with the abovementioned limitations which may have affected the accuracy of our results. Despite these barriers, our study is evidence that there are some clear differences between low risk GS 7 patients and high risk GS 7 patients at the genetic level and that these differences can be exploited to obtain a better understanding of their prognostic outcome. At the very least, our study offers a compelling stance that prognostic gene signatures have an important role to play in clinical diagnosis and treatment decisions for intermediate risk prostate cancer patients.

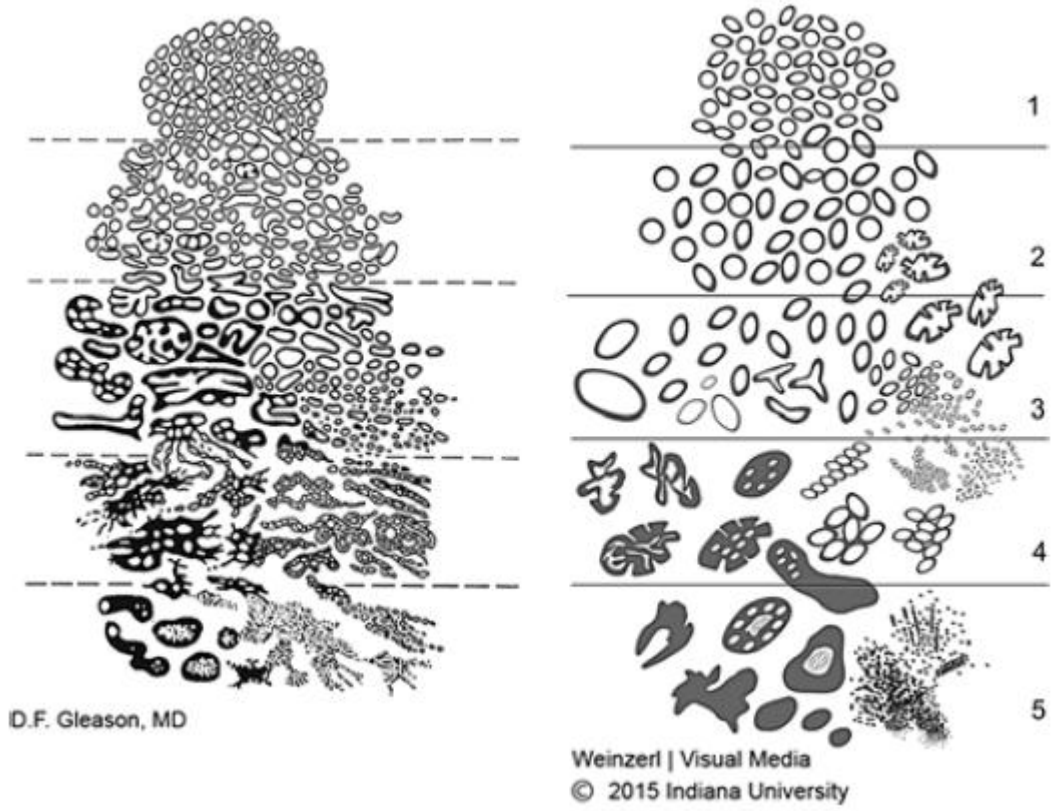
It is also worthwhile to highlight the success of our gene expression study using archival FFPE tumour tissue, a method that is commonly reported to

involve many difficulties both molecularly and technically. The greatest barrier in this study related to FFPE blocks was largely in the retrieval process, an issue that will face any Ontario research group planning to study archival FFPE tissue located in multiple healthcare locations. From a health economics standpoint, such barriers should be addressed to improve medical research efficiency, especially since it is all done in the interest of future patients' well-being. On that note, it is difficult to refute the fact that archived FFPE tumour tissue is and will be a valuable resource for large-scale research on prognostic gene signatures in the field of oncology. NanoString technology was also able to prove its worth in terms of producing high-quality quantification results on archival FFPE tissue, particularly from samples with extremely low amounts of RNA and without the use of any amplification steps. Our study contributes to the growing opinion that one can opt for NanoString's nCounter technology for high-quality and reliable gene expression analysis in lieu of conventional and perhaps outdated methods.

Our hope is that our research will contribute to the future development of a commercially available clinical assay specific to GS 7 intermediate risk prostate cancer patients that can better stratify them into appropriate treatment groups. The next step towards these goals is to test our signatures on a larger cohort of more recent GS 7 prostate cancer patients to both improve statistical power and its external validity.

TABLES AND FIGURES

Figure 1. Schematic diagram comparing original 1974 Gleason Scoring system with updated 2015 Gleason Grading System



(Epstein et al., 2016)

Figure 2. Schematic diagram of Gleason Scoring System after 2005 ISUP modifications

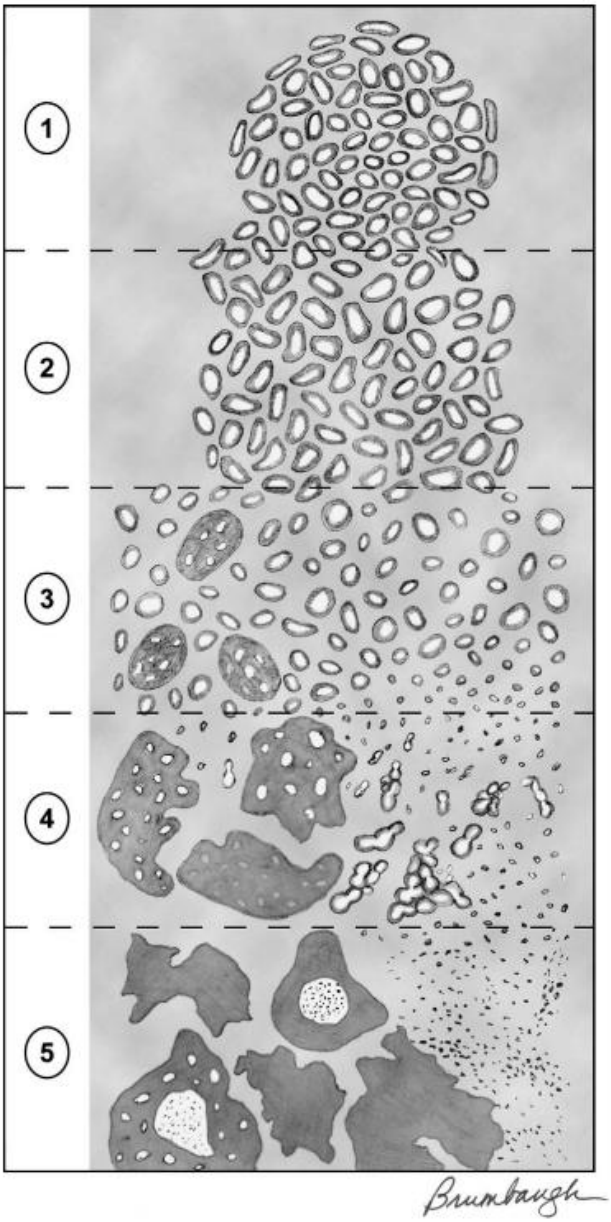
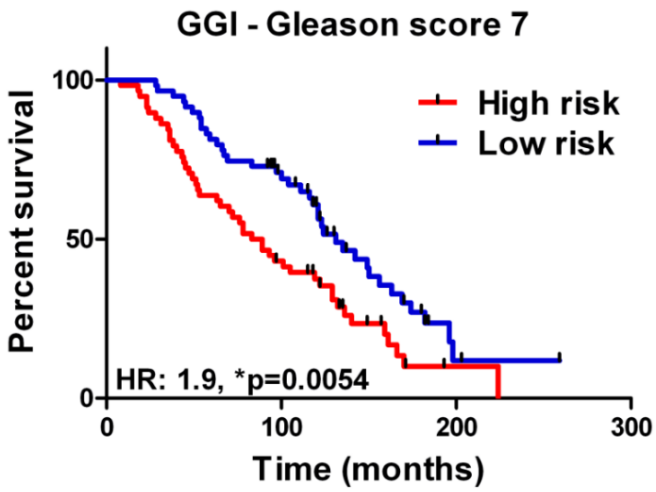
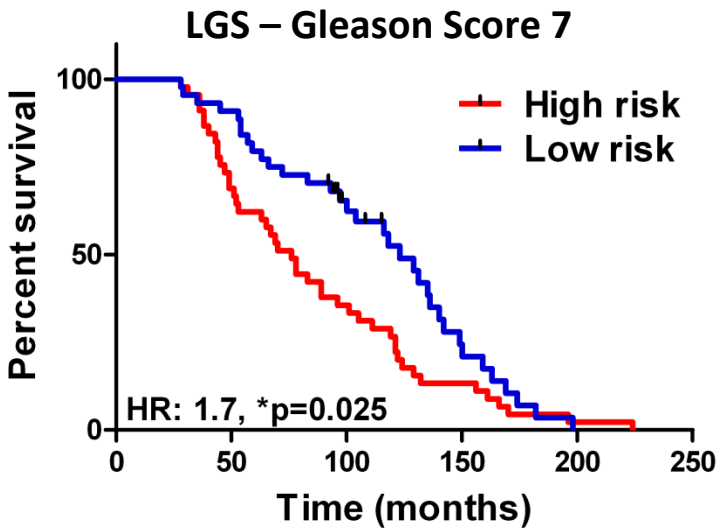


Figure 3. Stratification of GS 7 patients from SWW cohort using GGI signature



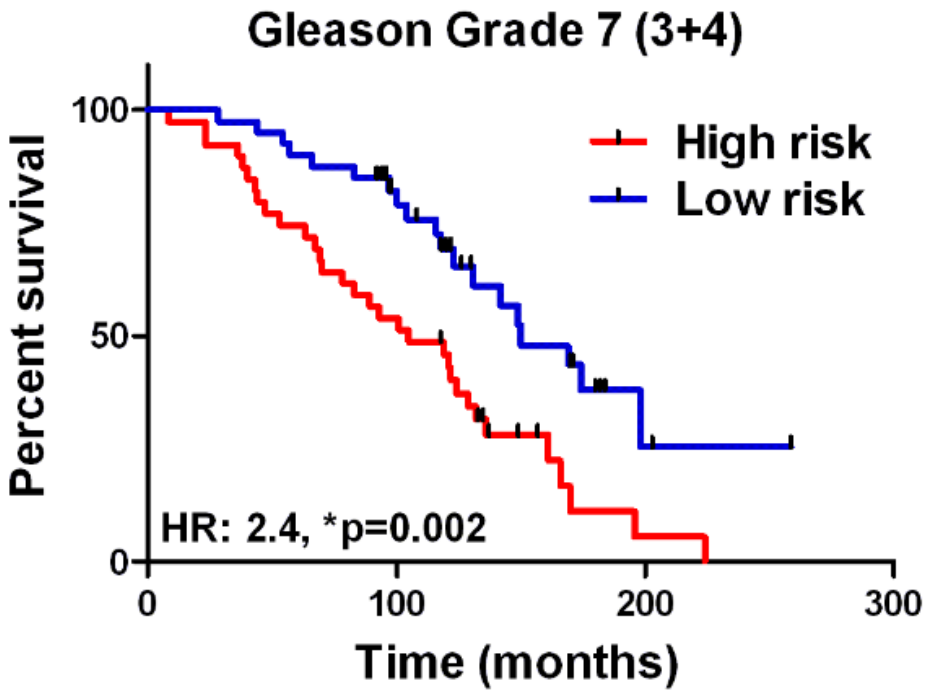
25 genes were found that differentiated between low (GS 6) and high (GS 8-10) grade GS tumours. Using these genes, GS 7 patients of the SWW cohort were accurately stratified into high and low risk (HR: 1.9, *$p<0.05$).

Figure 4. Stratification of GS 7 patients from SWW cohort using LGS signature



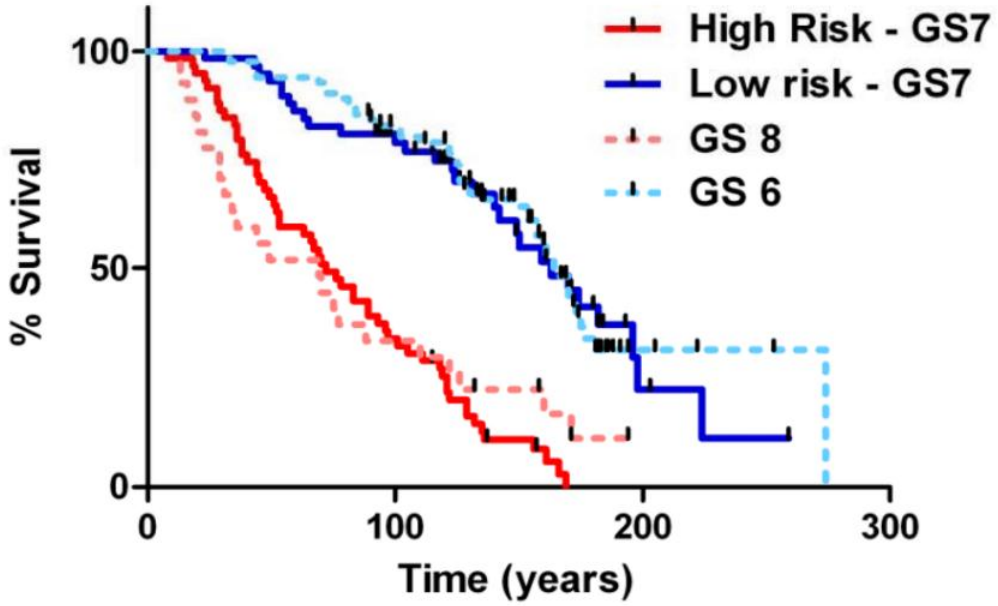
25 genes were found that differentiate between good (at least 10 year survival) and poor outcome (death before 2 years). Using these genes, GS 7 patients of the SWW Cohort were accurately stratified into high and low risk (HR: 1.7, *$p<0.05$).

Figure 5. Stratification of GS 7 patients from SWW cohort using a combined GGI+LGS signature



A combined index of the GGI and the LGS was able to stratify GS 7 patients of the SWW cohort more accurately into high and low risk. Dotted line indicates 10 year mark (HR: 2.7, *p<0.0001)

Figure 6. Stratification of GS 7 patients from SWW cohort using Network signature



88 genes were identified using a network based approach which accurately stratified GS 7 patients of the SWW cohort into high risk and low risk groups. Survival curves of low risk GS 6 and high risk GS 8 were superimposed onto the graph, showing high similarity to the two stratified GS 7 groups.

Figure 7. Survival curves of an untreated cohort of SWW patients

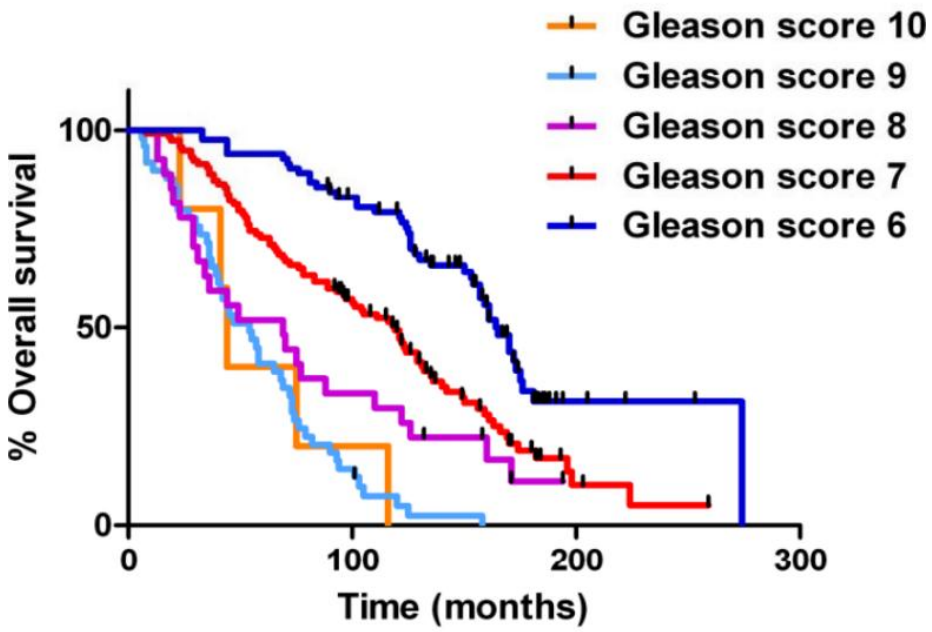


Figure 8. Sample accrual outlining the number of included and excluded samples

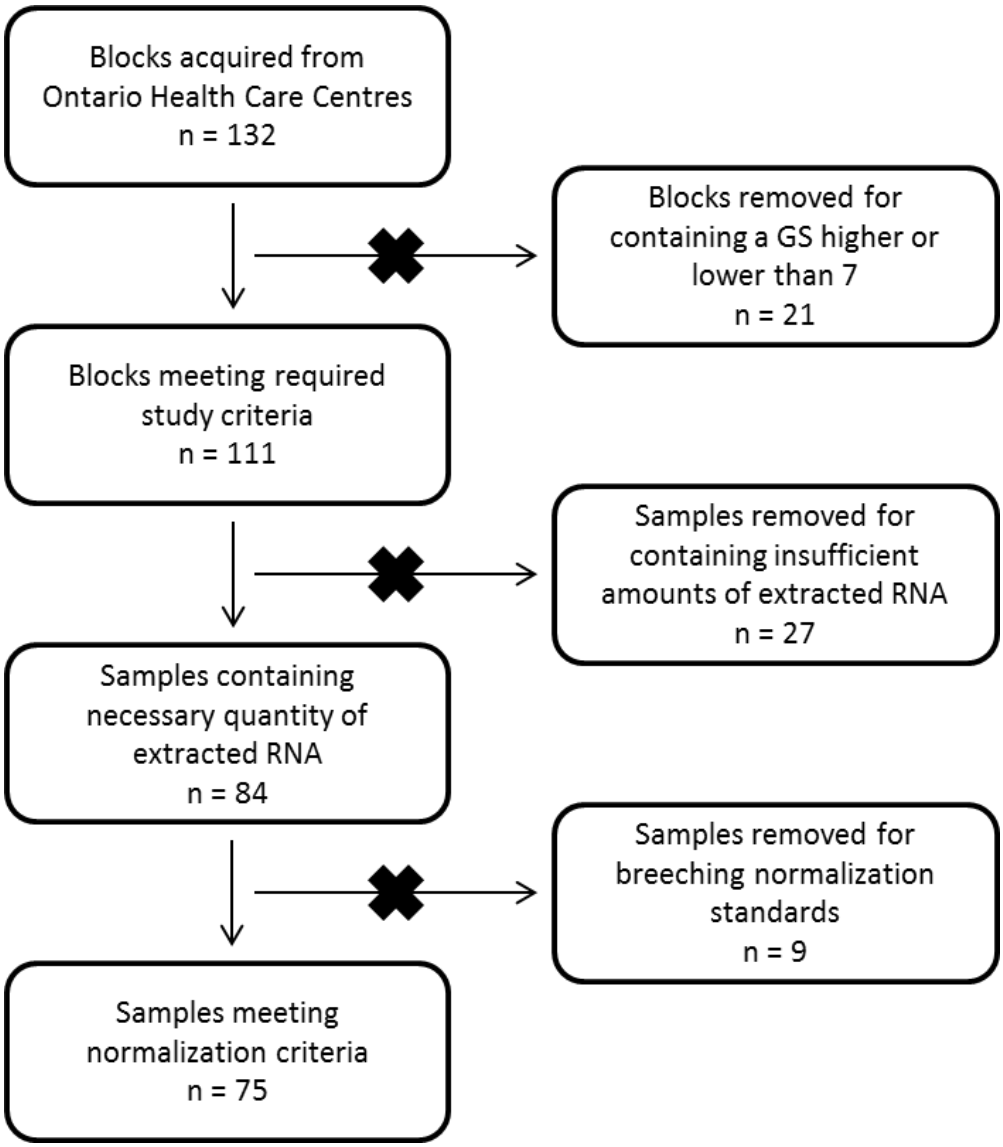


Figure 9. RNA extraction results comparing four different extraction kits

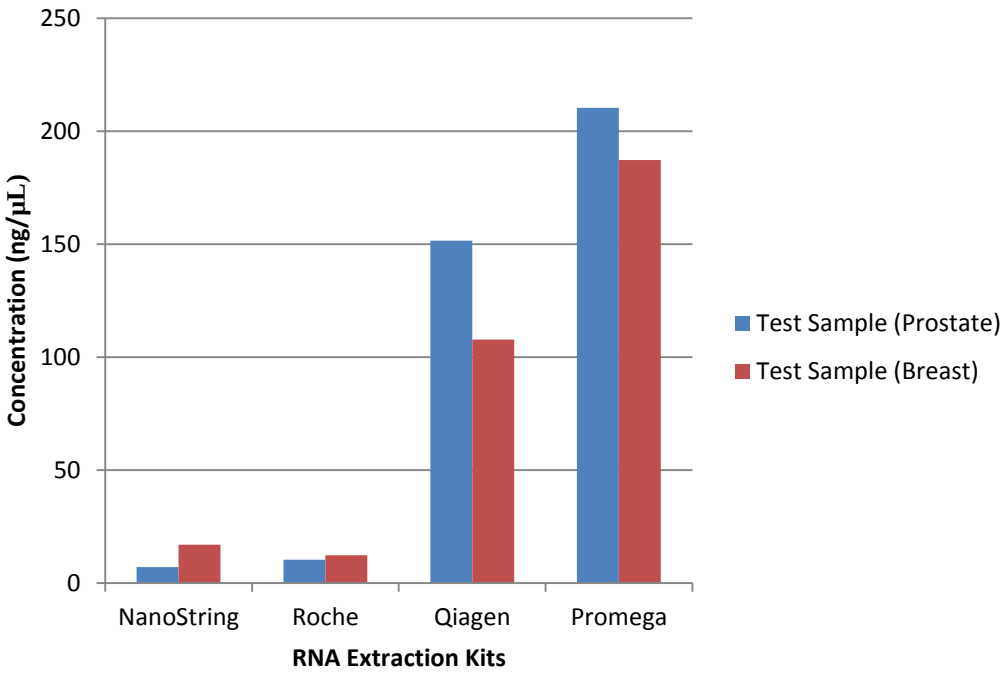


Figure 10. Positive and Negative Normalization on a $\log_{10}$ Scale of Final NanoString Analysis

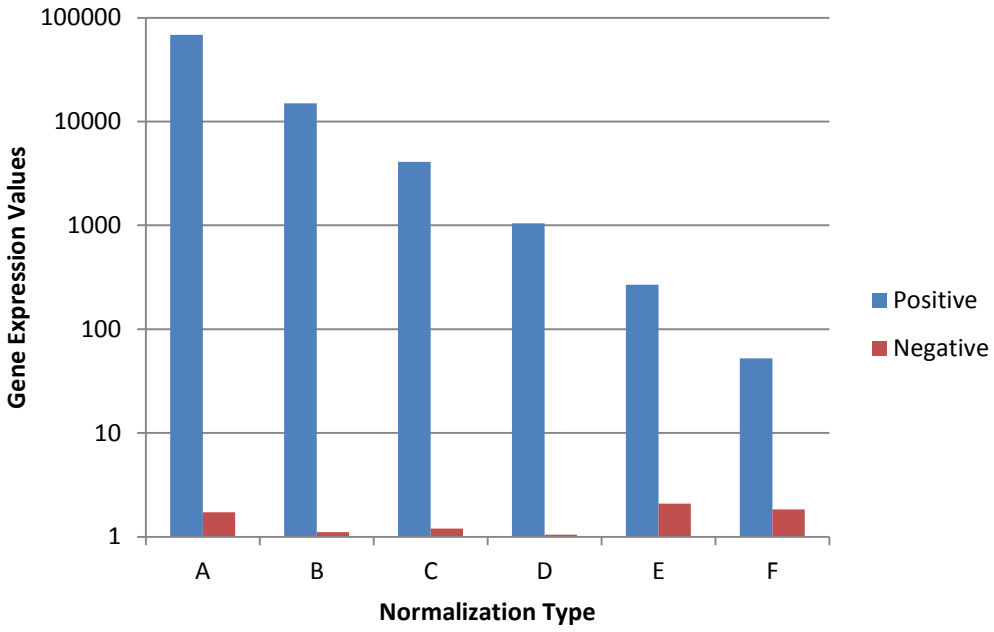


Figure 11. Bioanalyzer results outlining RIN Score of 12 prostate samples

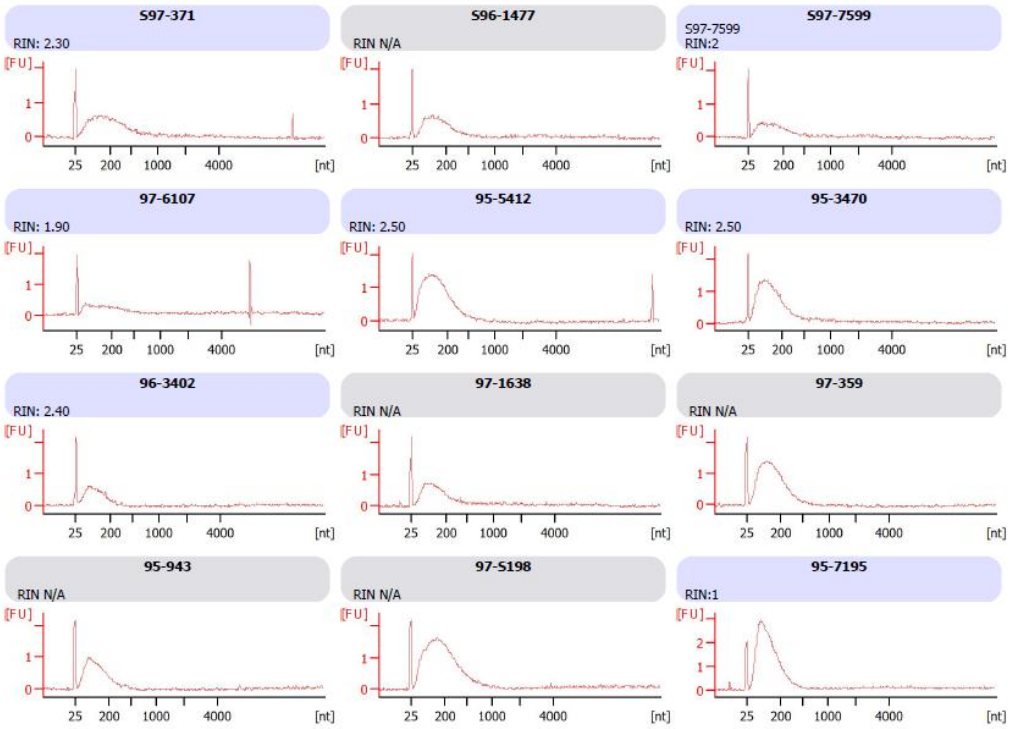


Figure 12. Example of two H&E stained scored needle-core prostate biopsy slides

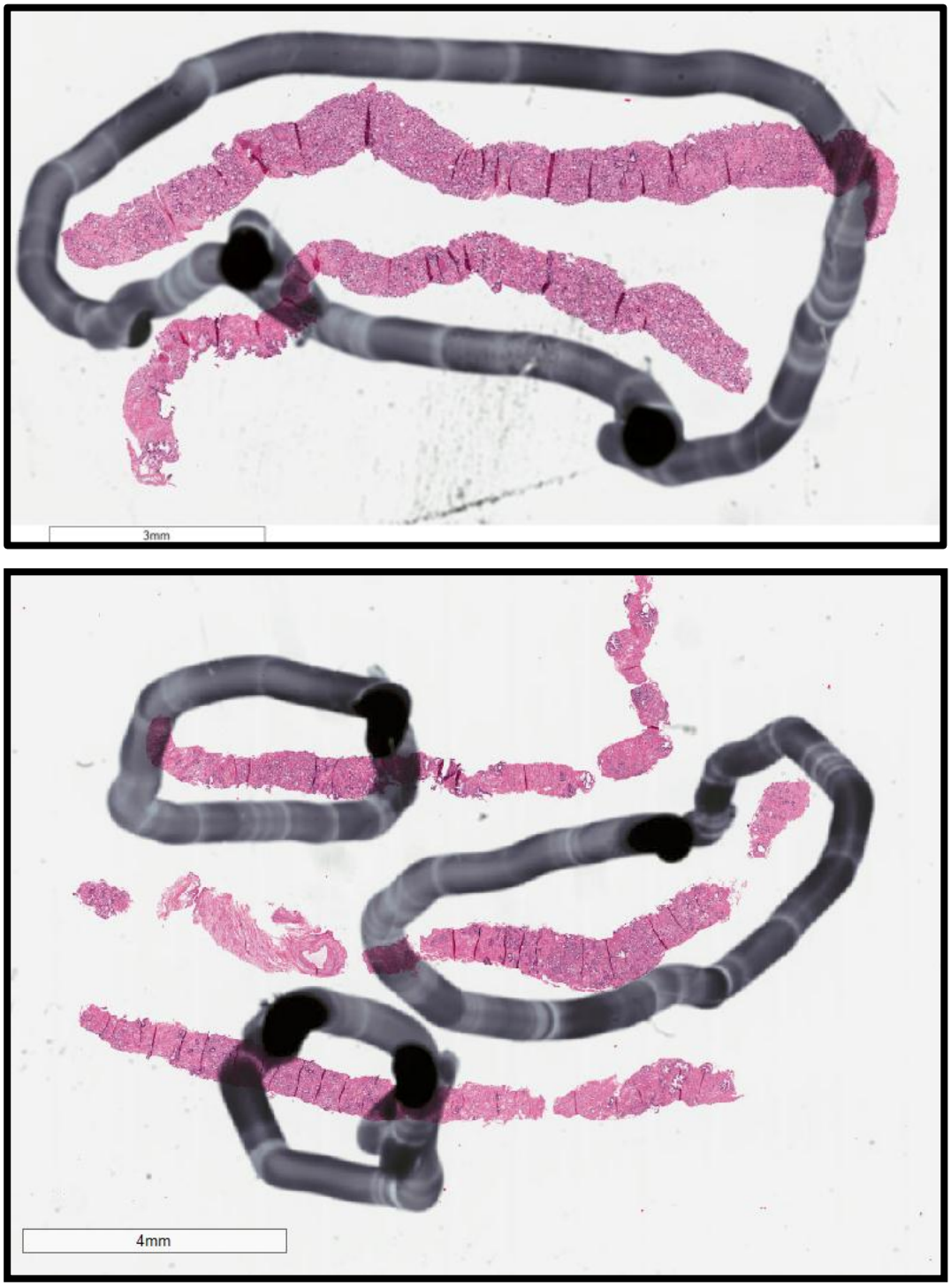


Figure 13. Morphology of intraductal carcinoma at 5X and 20X magnification

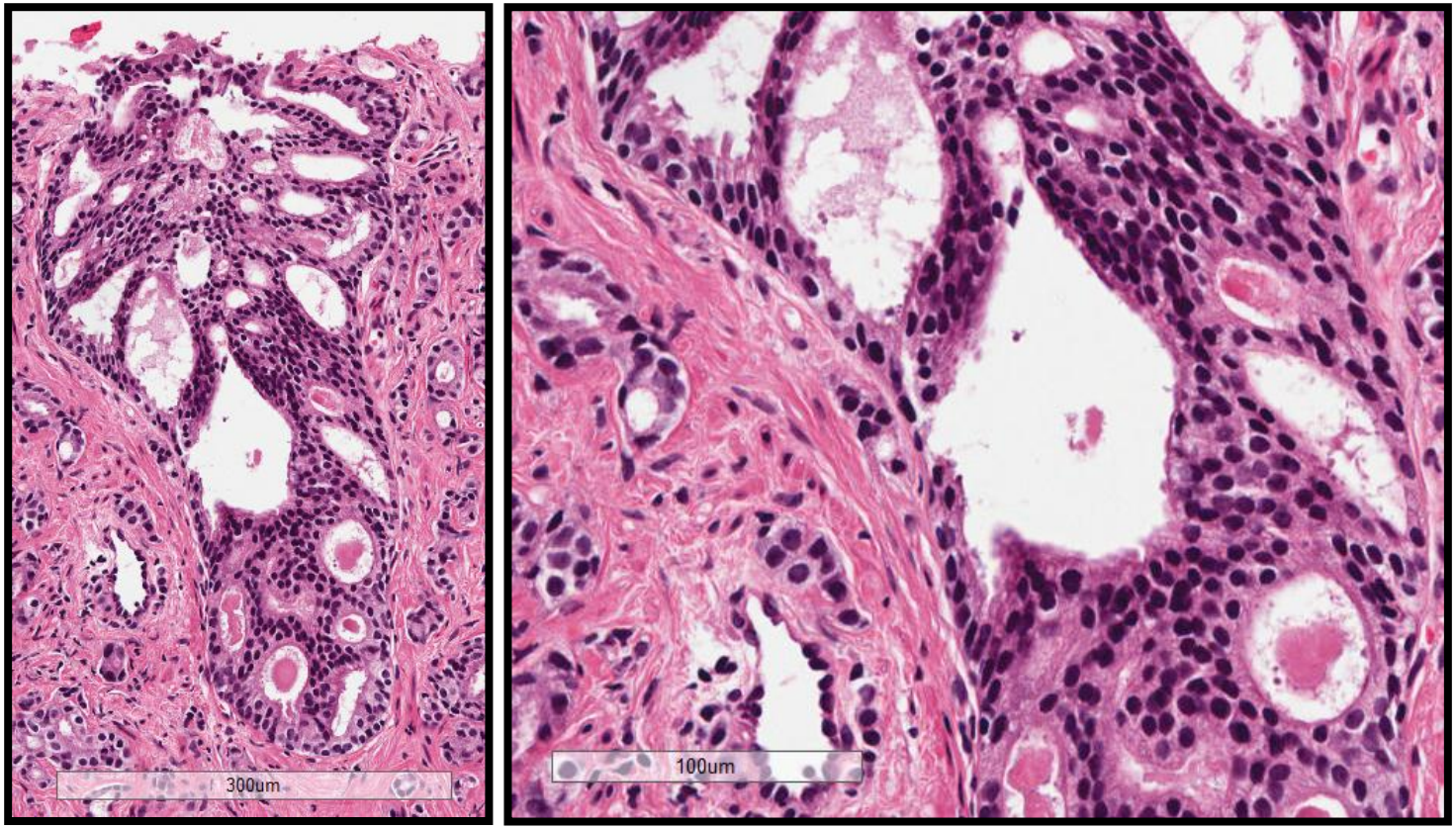


Figure 14. Morphology of cribriforming at 5X and 20X magnification

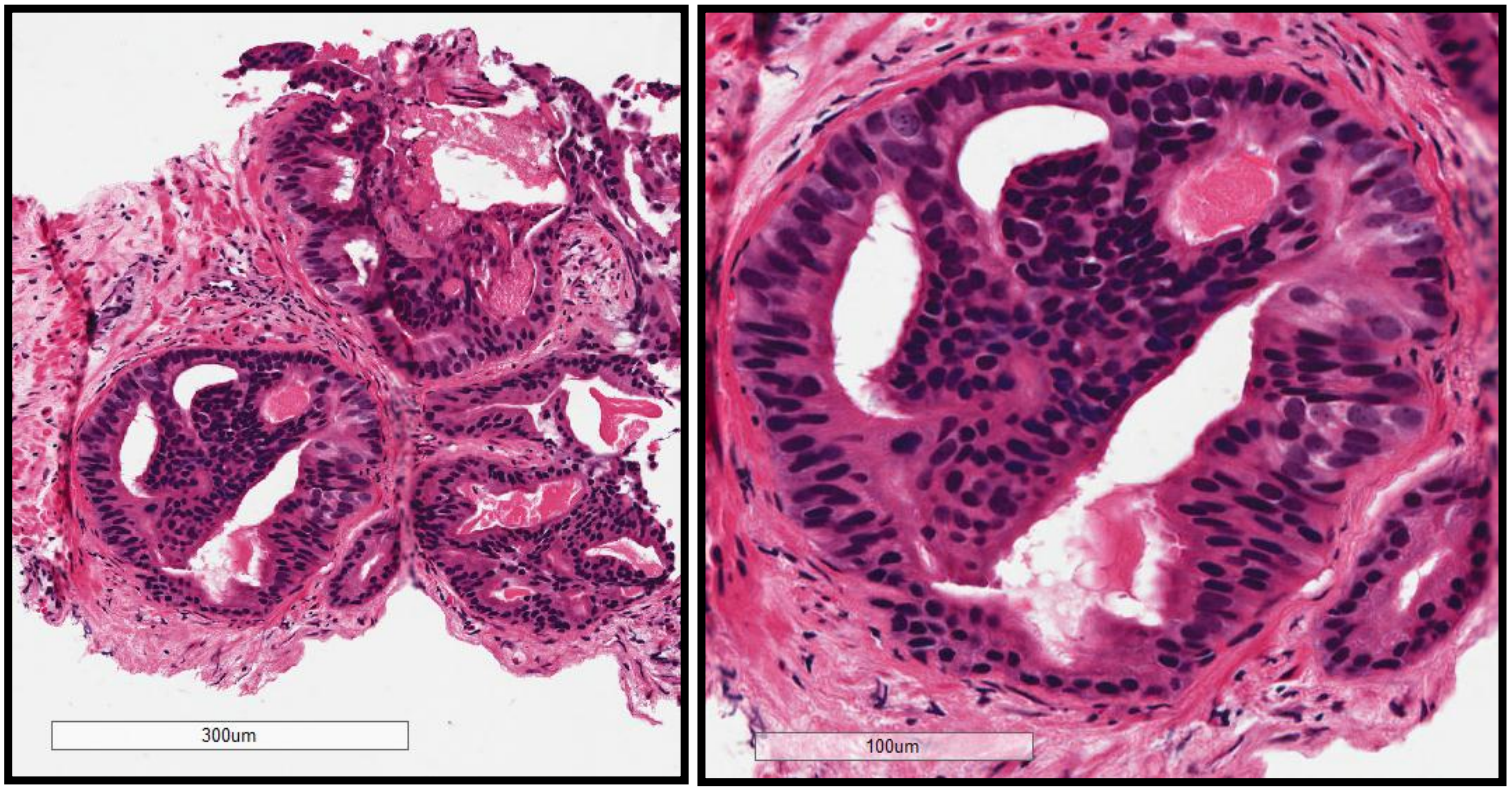


Figure 15. Morphology of Gleason grade 3 and Gleason grade 4 at 5X and 20X magnification

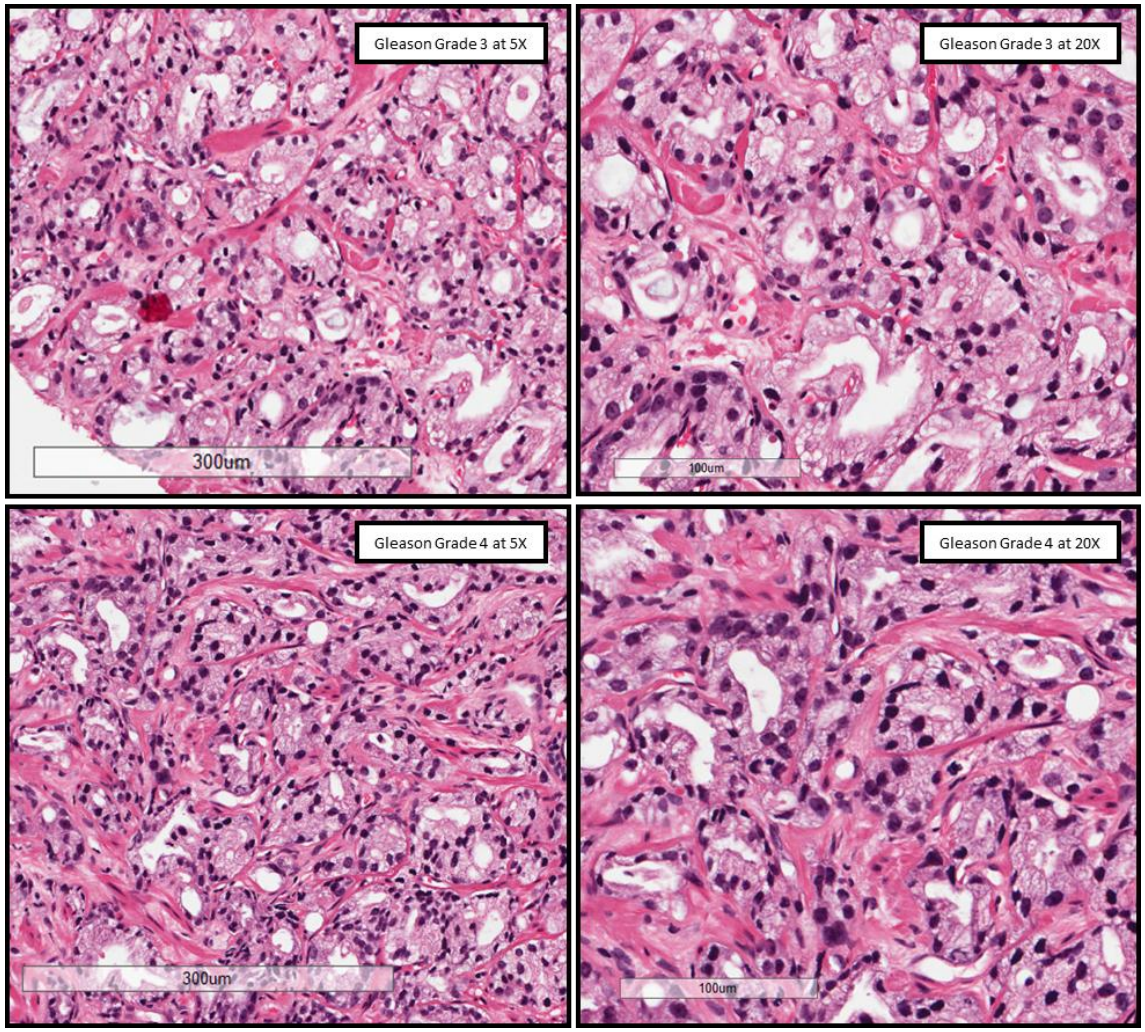


Figure 16. Log_2 expression values of housekeeping genes

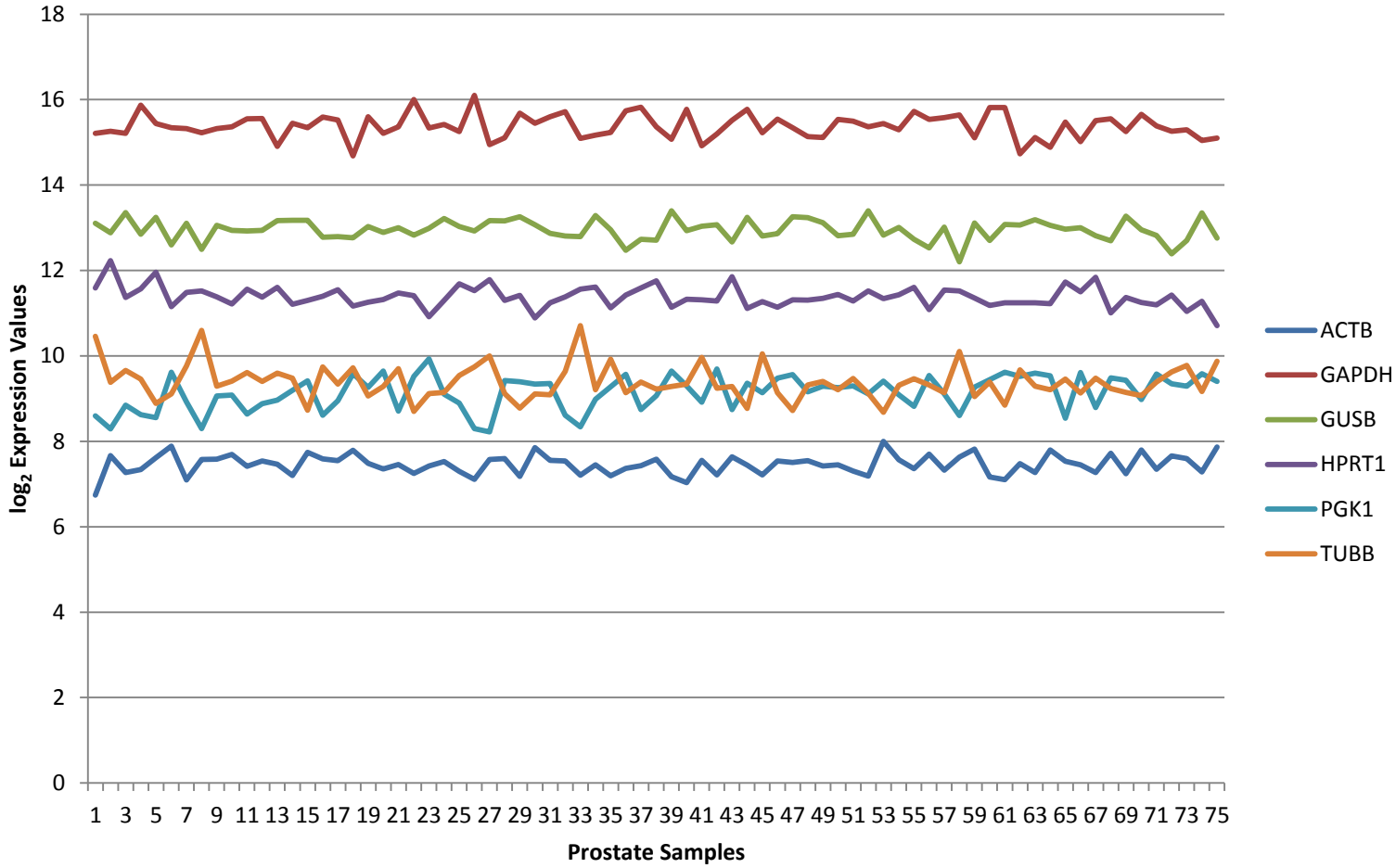


Figure 17. Network signature module map outlining intra- and inter-module interactions between 10 modules

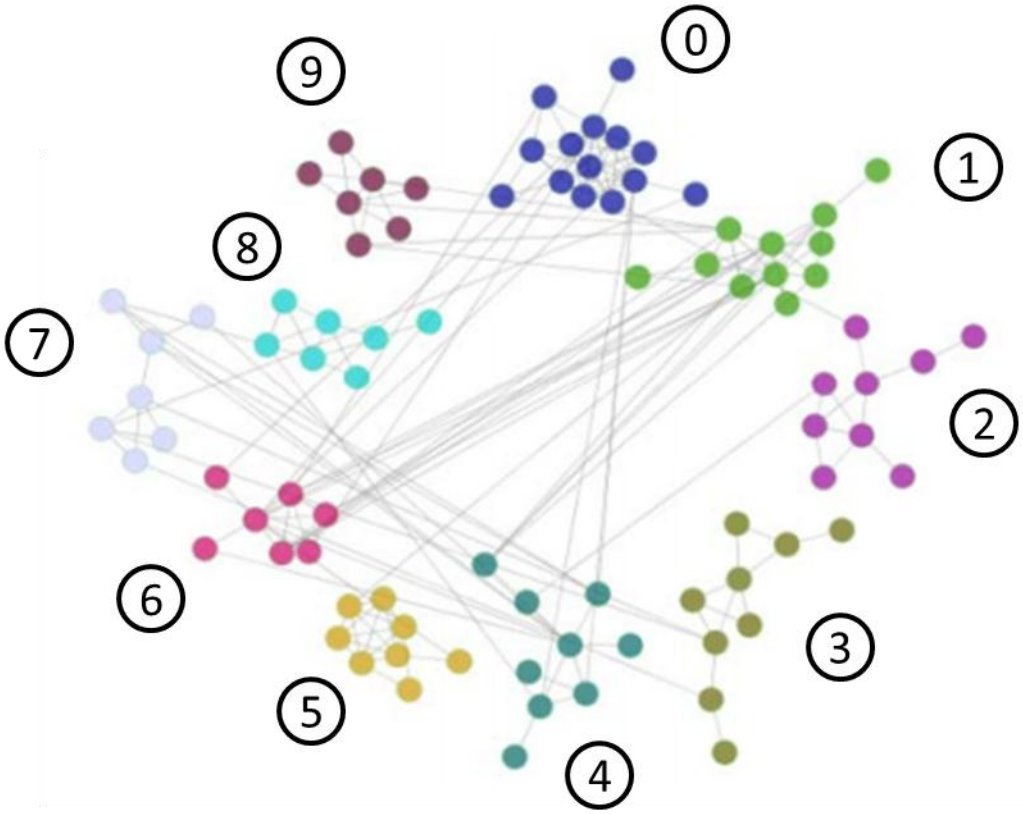
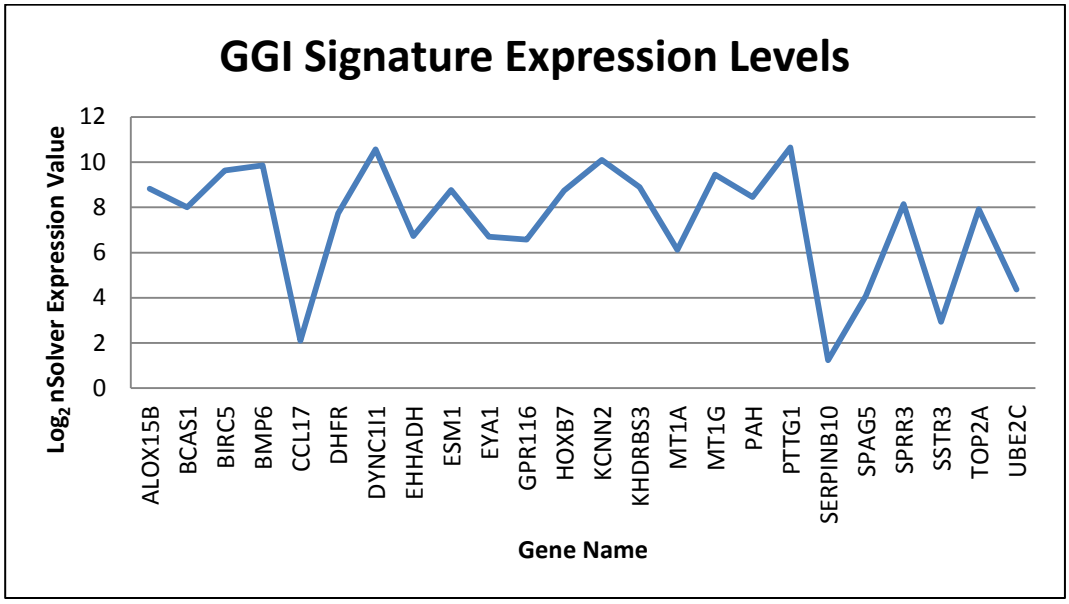


Figure 18. Log_2 Expression values of GGI and LGS signatures



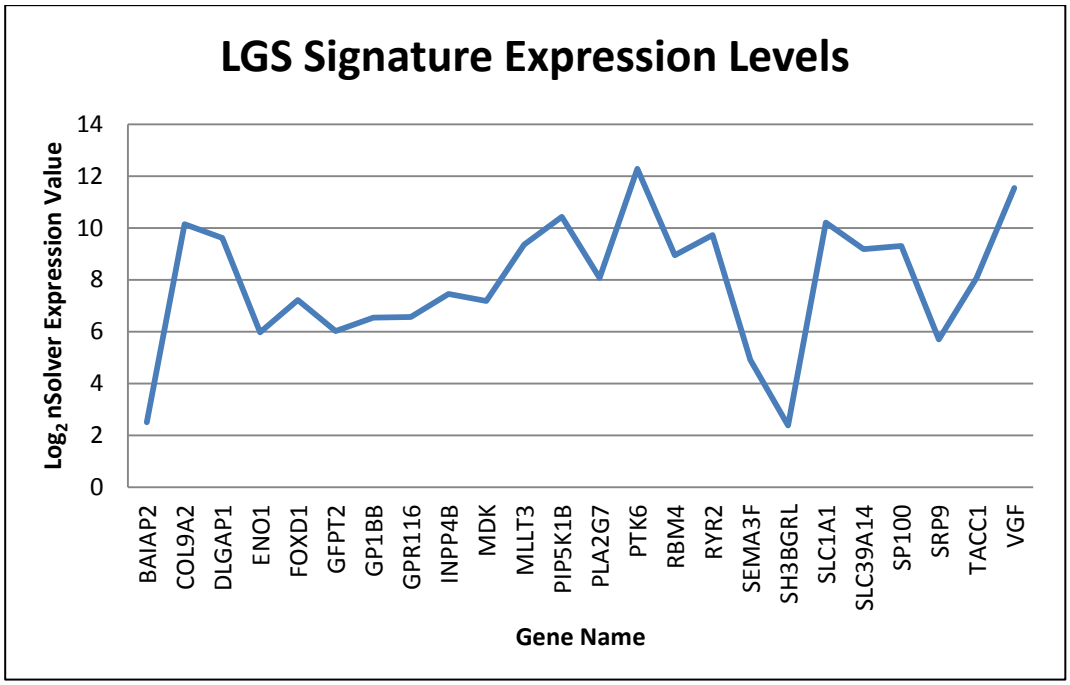


Figure 19. Log_2 expression levels of Network signature

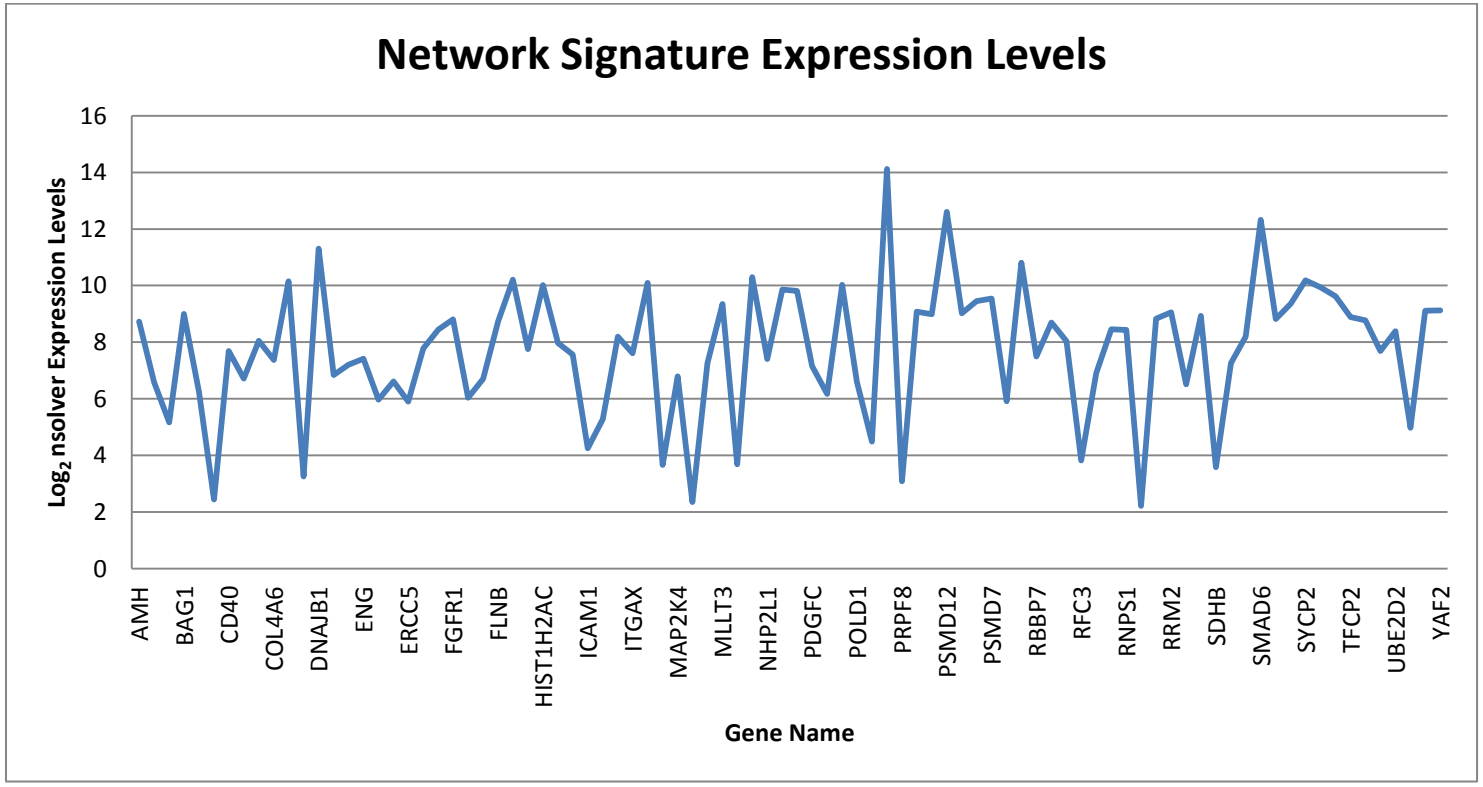


Figure 20. Expression levels of PTEN, TMPRSS2, and ERG

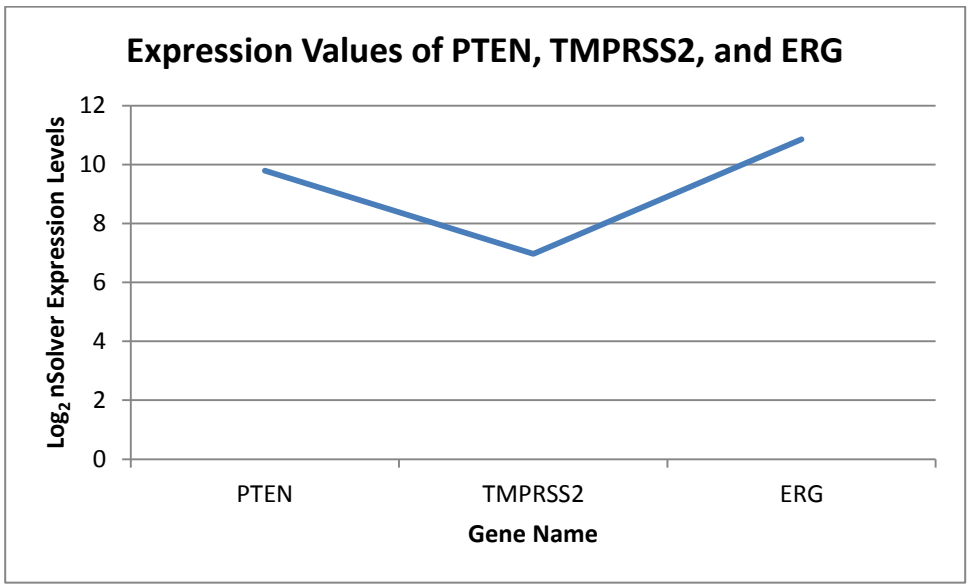


Table 1. Updated Gleason Scoring method

Traditional Gleason Score	New Prognostic Group
≤ 6	1 – only individual discrete well-formed glands
3 + 4 = 7	2 – predominantly well-formed glands with lesser component of poorly-formed/fused/cribriform glands
4 + 3 = 7	3 – predominantly poorly-formed/fused/cribriform glands with lesser component of well-formed glands*
4 + 4 = 8 3 + 5 = 8 5 + 3 = 8	4 – only poorly-formed/fused/cribriform glands; predominantly well-formed glands and lesser component lacking glands**; predominantly lacking glands and lesser component of well-formed glands**
9 & 10	5 – lacks gland formation (or with necrosis) with or without poorly formed/fused/cribriform glands*
*For cases with > 95% poorly-formed/fused/cribriform glands or lack of glands on a core or at radical prostatectomy, the component of < 5% well-formed glands is not factored into the grade **Poorly-formed/fused/cribriform glands can be a more minor component	

(Epstein et al., 2016)

Table 2. Risk Stratification for prostate cancer patients based on GS, PSA, and tumour stage

	T_{1-2} ← →			T_3
	PSA ≤ 10	PSA 10.1-20	PSA ≥ 20	
GI Score ≤ 6	(T_{2a})			
GI Score = 7				
GI Score ≥ 8				

Key
Low Risk
Inter. Risk
High Risk

(Rodrigues, 2012)

Table 3. Commercially available prognostic gene signatures for prostate cancer patients

Test Name	Eligibility Criteria	Sample Requirements	Number of Genes
OncotypeDX Prostate Cancer Test	Recently diagnosed early-stage prostate cancer GS 6 or less GS 7 (3+4), but only if tumour involvement less than or equal to 33% of biopsy mass PSA less than 20 Tumour stage of T1 or T2	FFPE needle core biopsies of prostate	12 cancer genes 5 housekeeping genes
Prolaris	Patients in Active Surveillance or have received radical prostatectomy	FFPE needle core biopsies of prostate 7 slides, 3-5 μm thickness with at least 0.5mm of tumour length	31 cell cycle progression genes 15 housekeeping genes
Decipher	Patients who have received radical prostatectomy	FFPE tissue from radical prostatectomy with highest Gleason grade	22 genes relating to various process of interest

Table 4. Outline of the uses of different commercially available RNA testing panels

	Decipher	**Oncotype Dx**	**Prolaris**
Cancer recurrence after RP (BCR)	-	Yes	Yes
Metastasis after RP	Yes	-	Yes
PCa-specific mortality after RP	Yes	-	Yes
PCa progression (PCa-specific mortality) in AS population	-	Yes	Yes

(Na, Wu, Ding, & Xu, 2016)

Table 5. Summary of 10 Network signature modules

Module	Biological Pathway	Hazard Ratio	P-value
0	Immune Signalling	1.7	2.8 E-8
1	Epigenetics	1.9	5.7 E-9
2	Protein Regulation	1.9	8.0 E-9
3	TGF-β, Integrin, Growth Factor receptor signaling	1.7	7.6 E-7
4	Immune Signalling	1.6	1.0 E-4
5	Transcription	1.7	1.2 E-6
6	Transcription	1.6	3.6 E-5
7	Epigenetics	1.6	5.4 E-6
8	TGF-β, Integrin, Growth Factor receptor signaling	1.6	3.5 E-5
9	TGF-β, Integrin, Growth Factor receptor signaling	1.5	1.7 E-5
Combination	N/A	3.9	3.1 E-15

Each module is associated with a specific biological pathway. Hazard ratios and p-values show that each module is significantly associated with patient outcome. A combination of the modules leads to greater association.

Table 6. H&E slide scoring results of final 75 samples

	Count	**Percentage**
Total Sample Size	75	100%
Gleason Score		
3 + 4	55	73.3%
4 + 3	20	26.7%
Biopsy Type		
Needle-core	72	96%
TURP	3	4%
Presence of Cribriforming		
Yes	48	64%
No	27	36%
Presence of Intraductal Carcinoma		
Yes	2	2.7%
No	73	97.3%
BCR		
Yes	35	46.7%
No	40	53.3%
Total Extent of Tumour (mm)		
1 – 10	31	41.3%
11 – 20	26	34.7%
21 – 30	11	14.7%
30 <	4	5.3%
N/A (TURP Biopsy)	3	4%

Table 7. RNA extraction results of final 75 samples using BioDrop

RNA Extraction	**Concentration (ng/µL)**
Mean	38.67
Median	28.43
Standard Deviation	29.2
Highest	154.00
Lowest	7.17
RNA A260/A280	**Value**
Mean	1.85
Median	1.85
Standard Deviation	0.06
Highest	2.06
Lowest	1.71

Table 8. Univariate Cox Regression Analysis Results on Primary Questions

				95% CI	
Signatures	**B**	**p value**	**HR**	**Lower**	**Upper**
GGI+LGS	-2.301	0.002	0.100	0.024	0.427
Network	-1.183	0.155	0.306	0.060	1.563
GGI	-1.725	0.000423	0.178	0.068	0.465
LGS	-0.520	0.362	0.595	0.195	1.817
M0	-0.297	0.346	0.743	0.401	1.378
M1	-0.681	0.006	0.506	0.311	0.824
M2	-0.158	0.678	0.854	0.404	1.804
M3	-0.605	0.006	0.546	0.354	0.844
M4	-0.108	0.685	0.898	0.533	1.512
M5	-0.372	0.145	0.690	0.418	1.137
M6	0.011	0.973	1.011	0.526	1.945
M7	0.543	0.013	1.722	1.124	2.638
M8	0.190	0.427	1.210	0.756	1.936
M9	0.242	0.481	1.274	0.650	2.497

Table 9. Univariate cox regression results on secondary questions

				95% CI	
Signatures	**B**	**p value**	**Exp(B)**	**Lower**	**Upper**
GGI+LGS & Network	-3.804	0.001	0.022	0.002	0.216
GGI+LGS & PTEN & TMPRSS & ERG	-0.213	0.179	0.808	0.592	1.103
Network & PTEN & TMPRSS & ERG	-0.197	0.220	0.821	0.599	1.126
GGI+LGS & Network & PTEN & TMPRSS & ERG	-0.272	0.162	0.762	0.520	1.115
Gleason Component (3+4 or 4+3)	0.086	0.806	1.090	0.546	2.177
Total Tumour Length	1.044	0.020	1.044	1.007	1.082

Table 10. Complete gene list of 141 genes

GGI	LGS	Housekeeping Genes	Additional Genes	Network Signature		
ALOX15B	BAIAP2	ACTB	ERG	AMH	ICAM1	PSMD8
BCAS1	COL9A2	GAPDH	PTEN	ANP32A	IL2RA	RBBP5
BIRC5	DLGAP1	GUSB	TMPRSS2	APCS	IL8	RBBP7
BMP6	ENO1	HPRT1		BAG1	IRF5	RBM5
CCL17	FOXD1	PGK1		BCR	ITGAX	REL
DHFR	GFPT2	TUBB		BMP4	KHDRBS1	RFC3
DNCI1	GP1BB			CCNE2	KHDRBS3	RING1
EHHADH	INPP4B			CD40	LMNB1	RNF2
ESM1	LRPAP1			CD44	MAP2K4	RNPS1
EYA1	MDK			COL3A1	MAPK8	RPL23
GPR116	MLLT3			COL4A6	MELK	RRM2
HOXB7	PIP5K1B			COL9A2	MLLT3	RUVBL1
KCNN2	PLA2G7			DNAJB1	MMP15	SAP30
KHDRBS3	PTK6			DNMT1	NCBP2	SDHB
MT1A	RBM4			DUSP9	NHP2L1	SHC3
MT1G	RYR2			ENG	OGDH	SIAH1
PAH	SEMA3F			ENO1	PCNA	SMAD6
PTTG1	SH3BGRL			EPRS	PDGFC	SMAD7
SERPINB10	SLC1A1			ERCC5	PHC2	STK17B
SPAG5	SLC39A14			FCER1G	PIK3R2	SYCP2
SPRR3	SP100			FGF4	POLD1	TERF1
SSTR3	SRP9			FGFR1	POLR2C	TFCP2
TOP2A	TACC1			FGFR2	PRIM1	TRAF2
UBE2C	VGF			FKBP1A	PRPF8	TRIM21
				FLNB	PSMB7	UBE2D2
				GADD45A	PSMD1	UBE2D3
				HDAC9	PSMD12	UGCG
				HIST1H2AC	PSMD14	YAF2
				HIST1H2BJ	PSMD6	
				HSPA2	PSMD7	

Table 11. Complete gene list of Network-based signature genes categorized into the respective 10 modules and associated outcome

Module 0	Module 1	Module 2	Module 3	Module 4	Module 5	Module 6	Module 7	Module 8	Module 9
Immune Signalling	Epigenetics	Protein Regulation	TGF-β, Integrin, Growth Factor receptor signaling	Immune Signalling	Transcription	Transcription	Epigenetics	TGF-β, Integrin, Growth Factor receptor signaling	TGF-β, Integrin, Growth Factor receptor signaling
Genes Associated with Poor Outcome as established in *in silico* analysis									
CCNE2	ANP32A	KHDRBS3	AMH	HSPA2	NCBP2	DNMT1	COL9A2	BCR	PHC2
PSMD1	APCS	SIAH1	COL3A1	MAPK8	NHP2L1	ENO1	DUSP9	FGF4	RING1
PSMD8	HDAC9	UBE2D2	ENG	SYCP2	RNPS1	POLD1	FLNB	PIK3R2	
RRM2	HIST1H2AC	UBE2D3	FKBP1A				TRAF2		
	HIST1H2BJ		MMP15						
	LMNB1		SMAD6						
	MELK								
	TERF1								
Genes Associated with Good Outcome as established in *in silico* analysis									
EPRS	RBBP7	BAG1	SMAD7	CD40	ERCC5	GADD45A	COL4A6	FGFR1	MLLT3
ICAM1	RUVBL1	DNAJB1	BMP4	FCER1G	POLR2C	PCNA	ITGAX	FGFR2	RBBP5
OGDH	SAP30	KHDRBS1	CD44	IL2RA	PRPF8	PRIM1	MAP2K4	PDGFC	RNF2
PSMB7		STK17B		IL8	RBM5	RFC3		SHC3	TFCP2
PSMD12		TRIM21		IRF5	RPL23				YAF2
PSMD14				UGCG					
PSMD6									
PSMD7									
REL									
SDHB									

Table 12. Pathways associates with the GGI Signature compared to pathways involved in 3 commercially available prognostic gene signatures

GGI Signature	Oncotype Dx	Prolaris	Decipher
Cell Cycle Progression	Cellular Organization	Cell Cycle Progression	Cell Cycle Progression
Cellular Organization	Proliferation		Cellular Organization
Proliferation	Stromal Response		Proliferation
Immune Response	Androgen Signaling		Immune Response

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