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A STUDY OF TWO MAJOR CHALLENGES IN PROSTATE CANCER: EFFECTIVE CHEMO-PREVENTION AND OVERCOMING RESISTANCE TO HORMONAL AND CHEMOTHERAPY

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A study of two major challenges in prostate cancer: effective chemo-prevention and overcoming resistance to hormonal and chemotherapy

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my beloved family

ABSTRACT

For men, over the course of a lifetime the risk of developing prostate cancer is 1 in 9. Both the illness itself and treatment affect quality of life in multiple aspects, including urinary problems, pain, and sexual dysfunction. Current clinical challenges in this field include: inevitable drug resistance to treatments, lack of accurate diagnostic and prognostic biomarkers, as well as no common chemoprevention strategies. The aim of this thesis is therefore to identify transcript alternations associated with drug resistance (Study I and Study II), to evaluate potential drug targets for prostate cancer treatment (Study III), and to estimate the preventive effect of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) against prostate cancer (Study IV).

Castration resistance and docetaxel resistance are two significant issues that arise during prostate cancer disease progression. We employed next generation RNA sequencing technology to compare *hormone*-resistant (Study II) and *docetaxel*-resistant (Study I) vs respective sensitive cell lines, aiming to discover a potential drug target with the capability of prolonging the duration of hormone and docetaxel treatments before the cancer cells becoming resistant. The results showed that a variety of transcript alterations were obtained during resistance development, including mutations, altered gene expressions, and fusion transcripts. These alterations might be associated with drug resistance in prostate cancer.

As a rationale for Study III, we hypothesized that proteins that have never been reported as mutated in prostate cancer might play an important role in cancer progression through their essential function of maintaining cellular stability in cancer cells. Upon mutation, these genes would induce severe cellular instability such that the cells could not survive, and these cells would be erased through natural selection during cancer growth. In Study III, we therefore evaluated a non-mutated protein in prostate cancer, GPR89A, as a potential drug target with possible anti-cancer function.

Low-dose aspirin has been recommended by the U.S. Preventive Services Task Force (USPSTF) to prevent cardiovascular disease and colorectal cancer. However, results published in scientific studies are controversial regarding prostate cancer. In Study IV, we assessed whether maintenance use of aspirin or other NSAIDs could reduce the risk of prostate cancer. Based on data from the Swedish Cancer Register, the Swedish Prescribed Drug Register and the Swedish Causes of Death Register (2005-2012), we conducted a nationwide cohort study and found a protective effect of aspirin and other NSAIDs against prostate cancer, especially after long-term intake.

In sum, this thesis identified or assessed alternative methods against prostate cancer through exploring molecular approaches to develop more effective treatment methods, and by attempting to reduce the prevalence of cancer cases through chemoprevention.

LIST OF SCIENTIFIC PAPERS

I. **Ma Y**, Miao Y, Peng Z, Sandgren J, Ståhl T, Huss M, Lennartsson L, Liu Y, Nistér M, Nilsson S, Li C. Identification of mutations, gene expression changes and fusion transcripts by whole transcriptome RNAseq in docetaxel-resistant prostate cancer cells. *Springerplus.* 2016; 5(1): 1861-1872.

II. **Ma Y***, Miao Y*, Peng Z, Sandgren J, Ståhl T, Lennartsson L, Nilsson S, Li C. Identification of mutations, expression alterations and fusion transcripts by next generation RNAseq in castration-resistant prostate cancer cell lines with possible clinical relevance. *Journal of Next Generation Sequencing & Applications*. 2017; 4:2. DOI: 10.4172/2469-9853.1000149

III. **Ma Y***, Liu Y*, Liu J, Peng Z, Bajalica-Lagercrantz S, Nilsson S, Li C. Evaluation of GPR89A protein as drug targer in prostate cancer. *Manuscript.*

IV. **Ma Y**, Brusselaers N. Maintenance use of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) and prostate cancer risk. *Prostate Cancer and Prostatic Disease.* 2018; 21:147-152.

* *Equal contribution.*

RELATED PUBLICATIONS

(Not included in this thesis)

I. **Ma Y**, Shen Q, Schelin M, Jöud A, Andren O, Fall K, Andersson SO, Li C, Valdimarsdottir U, Fang F. Risk of cardiovascular diseases and psychiatric disorders during diagnostic workups for prostate cancer patients. *Manuscript in prep*.

CONTENTS

LIST OF ABBREVIATIONS

5-ARIs	5-α Reductase Inhibitors
ADT	Androgen Deprivation Therapy
ALSYMPCA	Alpharadin in Symptomatic Prostate Cancer
AR	Androgen Receptor
ATCC	American Type Culture Collection
BPH	Benign Prostatic Hyperplasia
CAPS	The Cancer Prostate Sweden Study
cfDNA	Circulating free DNA
CI	Confidence Interval
COSMIC	Catalogue Of Somatic Mutations In Cancer
CRPC	Castration-Resistant Prostate Cancer
CTCs	Circulating Tumor Cells
DDD	Defined Daily Dose
DHT	Dihydrotestosterone
DRE	Digital Rectal Exam
ERSPC	the European Randomized Study of Screening for Prostate
FFPE	Formalin-Fixed Paraffin-Embedded
GATK	Genome Analysis Toolkit
GO	Gene Ontology
GWAS	Genome-Wide Association Studies
ICD	International Classification of Diseases
IR	Inhibition Rate
LHRH	Luteinizing Hormone-Releasing Hormone
mCRPC	Metastatic CRPC
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PCPT	the Prostate Cancer Prevention Trial
PCR	Polymerase Chain Reaction
PIA	Proliferative Inflammatory Atrophy
PIN	Prostatic Intraepithelial Neoplasia
PLCO	Prostate, Lung, Colorectal, and Ovarian cancer screening trial

PSA	Prostate-Specific Antigen
qPCR	Quantitative PCR
RCTs	Randomized Controlled Trials
REDUCE	the Reduction by Dutasteride of Prostate Cancer Events trial
RNAseq	RNA sequencing
RT-PCR	Reverse Transcription PCR
SELECT	the Selenium and Vitamin E Cancer Prevention Trial
SIR	Standardized Incidence Ratio
SNP	Single Nucleotide Polymorphism
TaxR	Docetaxel-Resistant
TaxS	Docetaxel-Sensative
TNM	Tumor Node Metastasis system
USPSTF	U.S. Preventive Services Task Force
UTR	Un-Translated Region

LIST OF ABBREVIATIONS OF *GENES*/PROTEINS

ABCA	ATP-Binding Cassette Subfamily A
ABCB	ATP Binding Cassette Subfamily B
ABCC	ATP Binding Cassette Subfamily C
ADK	Adenylate Kinase
AP2B1	AP-2 Complex Subunit Beta
ATM	ATM Serine/Threonine Kinase
ATP6V1E2	V-type Proton ATPase Subunit E 2
BCR	B Cell Antigen Receptor
BRAF	B-Raf Serine/Threonine-Protein
BRCA 1/2	Breast Cancer Associated protein 1/2
COX	Cyclooxygenase
CYP17	Cytochrome P450 17
DGKB	Diacylglycerol Kinase Beta
EGF	Epidermal Growth Factor
EGR1	Early Growth Response 1
EIF3D	Eukaryotic Translation Initiation Factor 3 Subunit D
ERG	ETS-Related Gene
ETS	ETS Family of Transcription Factors
FOXP3	Forkhead Box Protein 3
GCR	Glucocorticoid Receptor
GPR89A	Golgi pH regulator A
GPS2	G Protein Pathway Suppressor 2
GRK1	Rhodopsin Kinase
IL1RA	Interleukin 1 Receptor Antagonist
KDM3A	Lysine Demethylase 3A
KDM5B	Lysine Demethylase 5B
LDLR	Low-Density Lipoprotein Receptor
MAPs	Microtubule-Associated Proteins
MIC1	Microneme-Associated Protein 1
MIPOL1	Mirror-Image Polydactyly Gene 1 Protein

MPP2	MAGUK p55 Subfamily Member 2
MSR1	Macrophage Scavenger Receptor 1
MYC	Myc Proto-oncogene
MYH9	Myosin-9
NADK2	NAD kinase 2, Mitochondrial
NCOR1	Nuclear Receptor Corepressor 1
NF-κb	Nuclear Factor Kappa B
PARP	Poly(ADP-Ribose) Polymerase
PIK3CD	Phosphatidylinositol 4,5-bisphosphate 3-kinase Catalytic Subunit Delta Isoform
PRKACB	cAMP-dependent Protein Kinase Catalytic Subunit Beta
PRKD1	Serine/Threonine-Protein Kinase D1
PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and Dual-specificity Protein Phosphatase
RB	Retinoblastoma
RERE	Arginine-Glutamic Acid Dipeptide Repeats Protein
SAMD8	Sphingomyelin Synthase-Related Protein 1
SND1	Staphylococcal Nuclease Domain-Containing Protein 1
SPDL1	Spindly-Like Protein 1
SPINK1	Serine Peptidase Inhibitor Kazal Type 1
SPOP	Speckle Type BTB/POZ Protein
STK3/4	Serine/Threonine-Protein Kinase 3/4
TAF15	TATA-Binding Protein-Associated Factor 2N
TFDP1	Transcription factor Dp-1
TLR	Toll-Like Receptors
TMPRSS2	Transmembrane Protease, Serine 2
VCL	Vinculin

1 INTRODUCTION

For men, prostate cancer is the most common cancer in Europe and the US, and the second leading cause of cancer death.[1] Adenocarcinomas which originate from prostatic epithelial cells, are the most common cancer subtypes (95%).[2] Prostate cancer is relatively less aggressive than other types of cancer. And patients have a high risk of dying from causes other than prostate cancer, such as cardiovascular diseases, external causes and diseases related to pulmonary circulation.[3]

Prostate cancer usually exhibits no symptoms during early course and grows slowly for years before any indications of the disease present themselves. Later symptoms include frequent and urgent urination, difficulty starting and controlling urination, pain or burning during urination, and sexual dysfunction. The most frequent metastasis locations are the bone, lymph nodes and brain. Bone pain is a late-phase symptom that occurs after the cancer has metastasized.

Current clinical challenges regarding prostate cancer include inevitable drug resistance to treatments, lack of accurate diagnostic and prognostic biomarkers, as well as no common chemoprevention strategies. As outlined in Figure 1, below, this thesis attempts to address a broad spectrum of prostate cancer issues, including its initiation, progression, metastatic development, and treatment resistance. The goals of this thesis are to identify transcript alternations associated with drug resistance (Study I and Study II), evaluate potential drug targets for prostate cancer treatment (Study III), and estimate the preventive effect of aspirin and other NSAIDs against prostate cancer (Study IV).

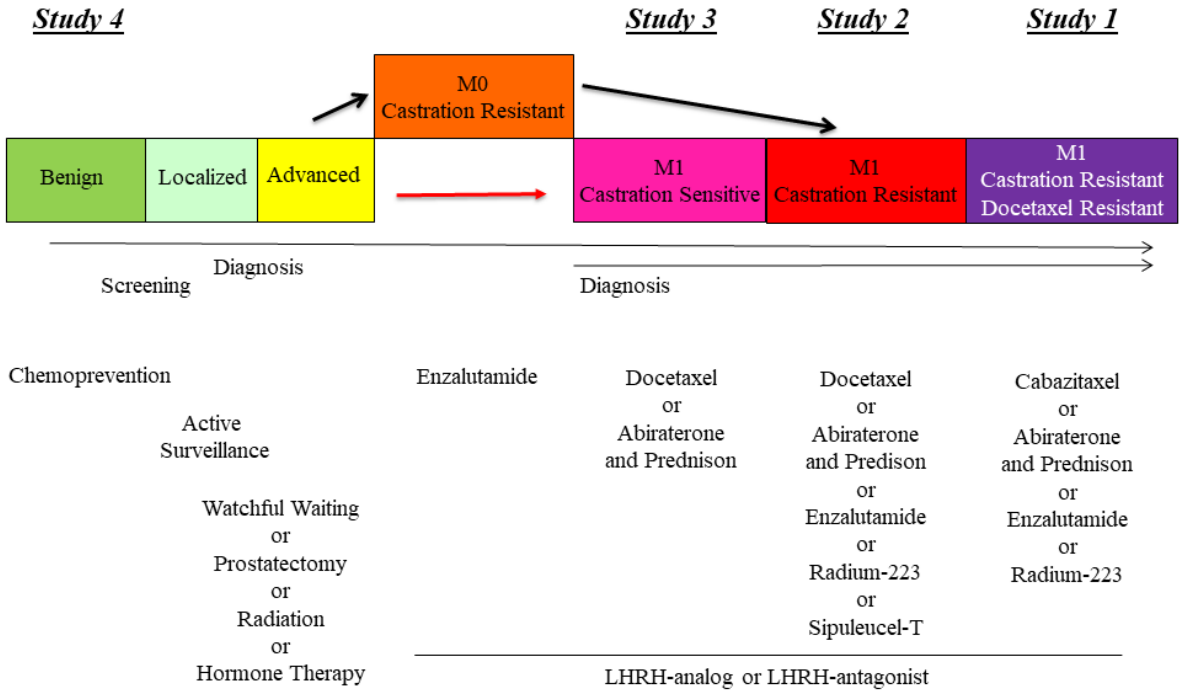

Figure 1 Prostate cancer disease continuum and corresponding treatments, as well as the overall outline of the thesis. *M: Metastatic.*

1.1 ETIOLOGY AND MOLECULAR BACKGROUND

The etiology of prostate cancer remains debated, although a number of factors are commonly believed to be associated with elevated risk of cancer development to date, including age, family history, lifestyle, and race. Prostate cancer occurs mainly in older men (99.9% of patients are over 50 years of age).[4] A family history of prostate cancer in both an individual's father and brothers increases the risk of developing the cancer by 2.3-times, as compared with men without a family history of prostate cancer.[5] A history of breast cancer in close female relatives is also significantly associated with a 1.22-fold increase in the risk of developing prostate cancer.[5] A higher number of relatives with prostate cancer history or with a history of having been diagnosed with the cancer at young age may further increase an individual's risk. Lifestyle factors such as regular exercise or a diet with low calcium intake have been found to be linked to decreased risk in developing prostate cancer. The incidence and mortality rates in different ethnic groups are differ significantly.[6]

Genetics is also suggested as a primary cause of prostate cancer. The contribution of genetic factors was found to constitute 58% of the risk of developing prostate cancer.[7] Genome-wide association studies (GWAS) have found weak to modest factors (more than 90 common single nucleotide polymorphisms (SNPs)) contributing to the development of prostate cancer, which taken together are estimated to explain one third of the cancer risk.[8] Other factors such as epigenetics or environmental causes also contribute to susceptibility of prostate cancer.

Genomic abnormalities include chromosomal loss or gain, gene amplification, mutations, and fusions (Figure 2). Prostate cancer has a relatively low rate of genomic alternations compared with other cancers, *e.g.*, 7-15 times lower than small cell lung cancer and melanoma.[9] Androgen receptor (*AR*) is the most commonly altered gene in metastatic prostate cancer, which leads to resistance to hormone treatment. *PTEN* (Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and Dual-specificity Protein Phosphatase) inactivation by copy number loss, deletions, or mutations happens in approximately 40% of prostate cancer.[10] *RB* (Retinoblastoma) loss and *p53* mutation are two other common types of genetic alterations in prostate cancer with 28% and 40% prevalence respectively.[11] *SPOP* mutation and *SPINK1* overexpression is also common, occurring in about 10% of prostate cancer (*SPOP*, Speckle Type *BTB/POZ* Protein, transcriptional repression activities; *SPINK1*, Serine Peptidase Inhibitor Kazal Type 1, trypsin inhibitor).[12, 13] DNA repair pathway deficiency, including *BRCA1* (breast-cancer–associated protein 1), *BRCA2* (breast-cancer–associated protein 2) and *ATM* (*ATM* Serine/Threonine Kinase), was discovered in about 20% of prostate cancer cases, which makes *PARP* (Poly(ADP-Ribose) Polymerase) a potential drug target.

ETS (*ETS* transcription factor) is a transcription factor family participating in cell differentiation, cell cycle, migration, proliferation, apoptosis and angiogenesis, as well as cancer progression. *ETS* family members were discovered as fusions with 5' un-translated region (UTR) of *TMPRSS2* (Transmembrane Protease, Serine 2) and its expressions were

highly up regulated as a consequence.[14] Among the *ETS* family, *TMPRSS2-ERG* (*ETS*-related gene) fusion is the most common rearrangement and has been found in nearly half of all prostate cancer samples.[15-17] *ETS* fusion can be detected in high grade prostatic intraepithelial neoplasia (PIN) and is considered to be associated with carcinogenesis instead of metastasis progression.[18-20]

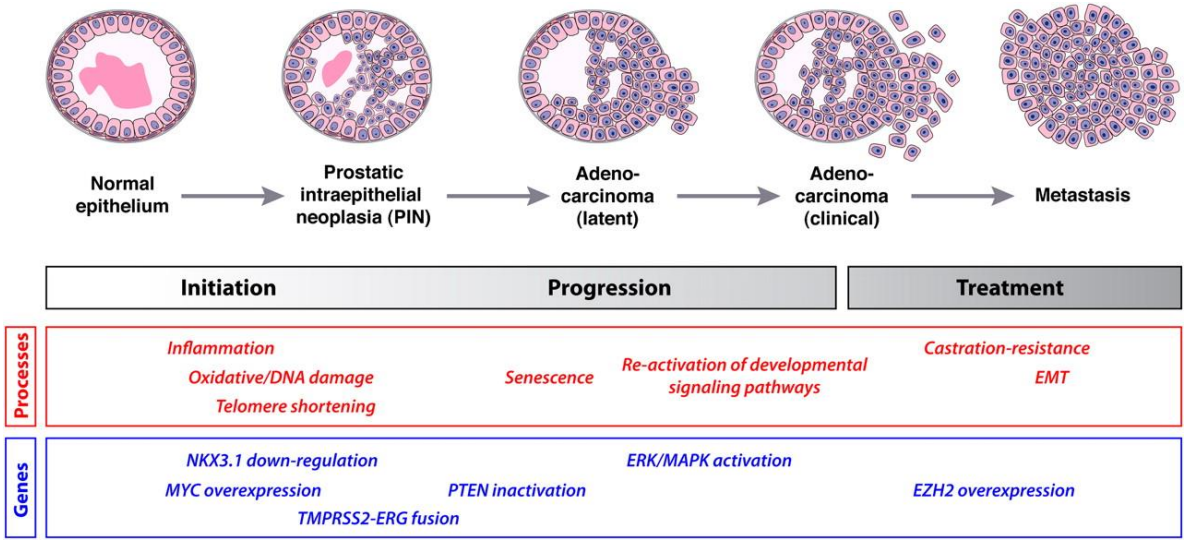

Figure 2 Progression pathway and involved processes and dysregulated genes in human prostate cancer. *(From: Molecular genetics of prostate cancer: new prospects for old challenges, Michael M. Shen and Cory Abate-Shen, 2010). EMT: Epithelial–Mesenchymal Transition.*

1.2 PREVENTION OF PROSTATE CANCER

Cancer is the second most common cause of mortality throughout the world. Many treatment methods, such as surgery, radiation, and chemotherapy have been developed to manage cancer. However, cancer is not easy to eliminate for multiple reasons, including but not limited to the issues of drug resistance or lack of powerful targeting strategies. Prostate cancer is a heavy burden in the lives of many men. The rationale for chemoprevention of prostate cancer is based on its high incidence, long-term disease course, and the low quality of life that results from the disease.

Cancer prevention is defined as taking action to lower the risk of developing cancer through healthy lifestyle choices (diet and exercise), avoiding exposure to cancer-causing substances, and taking vaccines or medicines (chemoprevention). Chemoprevention is a method to delay the onset of cancer through the intake of natural or synthetic agents. At present there are no common agents recommended by authorities for preventing prostate cancer, although many interventions have been proposed and investigated as preventive agents, including 5-α-reductase inhibitors (5-ARIs), NSAIDs, statins, and dietary supplements.[21]

5-α reductase inhibitors are the most studied chemopreventive agents against prostate cancer to date. 5-α reductase enzyme converts testosterone to dihydrotestosterone (DHT) which functions in the prostate and is involved in prostate cancer progression. The observation, that men with low 5-α reductase activity have less prostate cancer incidence, provides the rationale for using 5-ARIs to prevent prostate cancer.[22] A well-designed randomized controlled trials (RCTs), the Prostate Cancer Prevention Trial (PCPT), was designed to measure the preventive effect of the selective type two 5-ARI finasteride in men who were at low risk for prostate cancer and older than 55 years. The results showed that finasteride arm illustrated a reductive prevalence compared to placebo arm. However, the risk for intermediate and high grade cancers in finasteride group was apparently higher than the control group.[23] The REduction by DUtasteride of prostate Cancer Events (REDUCE) trial assessed the preventive effect of dutasteride and found that dutasteride arm showed decreased prostate cancer incidence and no statistically significant differences of high-grade cancer.[24] These 5-ARIs need further investigations until it can be concluded definitively whether finasteride induces high-grade prostate cancer.

Statins seem promising in the prevention of advanced prostate cancer based on a meta-analysis, which analyzed six large RCTs and 13 observational studies. These consistent findings supporte the argument that statins might be essential in the prevention of high-grade prostate cancer. A large number of dietary supplements have also been proposed and measured for their preventive potential in prostate cancer. These supplement reagents include selenium, vitamin E, vitamin A, vitamin C, multivitamins, green tea, calcium, folic acid and others. The best studied dietary supplements are selenium and vitamin E. One randomized trial, the Selenium and Vitamin E Cancer Prevention Trial (SELECT), recruited more than 35,000 men and assigned them randomly to four groups: selenium arm, vitamin E arm, selenium and vitamin E arm and placebo arm. However, the results showed no risk difference in incidence of prostate cancer among all arms.[25] Other dietary supplements have also been evaluated by observational studies and RCTs but none of them have provided adequate evidence and authorities have not recommend any as preventive agents against prostate cancer.

1.2.1 Inflammation in prostate cancer

Chronic inflammation is the cause of about 20% of all cancer in adults.[26, 27] Growing evidence finds that chronic or recurrent inflammation plays an important role in prostate cancer initiation, development, progression and metastasis.[28] Biopsies in several prostate diseases show signs of inflammation, including prostatitis, benign prostatic hyperplasia (BPH), prostate cancer “risk lesions” (proliferative inflammatory atrophy (PIA) and PIN), and prostate cancer.[29-31] Prostatic Intraepithelial Neoplasia, which presents as abnormal epithelial cell structure around inflammatory cells, was accepted as a prostatic adenocarcinoma precursor, while prostatitis and PIA were proposed but have not been widely accepted as precursors.[32-35] Chronic inflammation can generate PIA lesions and probably

develop to PIN and/or prostate cancer directly.[36, 37] The clinical trial REDUCE found that 80% of prostate cancer patients had more or less inflammation in biopsy tissues.[38]

Various potential sources for prostatic inflammation include, but are not limited to, urine reflux, dietary factors, estrogens, and direct infection (bacteria and viruses, such as human papillomavirus, herpes simplex virus type 2, cytomegalovirus, and human herpes virus-8).[35] Inflammatory genes relating to prostate cancer risk have also been discovered as mutations or other variants, including: *MSR1* (macrophage scavenger receptor 1), *TLR* (toll-like receptors), *MIC1* (Microneme-associated protein 1) and *IL1RA* (Interleukin 1 Receptor Antagonist).[39-42] The Cancer Prostate Sweden Study (CAPS) found a SNP in *TLR4* (11381G/C) to be associated with a 39% higher risk of early prostate cancer. However, a follow-up study of a North American population found eight other SNPs in *TLR4* while the 11381G/C variant did not show any association with prostate cancer. This likely indicates that SNPs differ among different populations.[39, 40]

1.2.2 Aspirin/NSAIDs and prostate cancer prevention

Nonsteroidal anti-inflammatory drugs are a family of drugs that can eliminate pain and reduce fevers and inflammatory responses. Most NSAIDs can reversibly inhibit cyclooxygenase (COX) enzyme activity on both COX-1 and COX-2.[43] Cyclooxygenase, namely prostaglandin-endoperoxide synthase, is an enzyme family that is responsible for prostanoid formation. COX-1 is constitutively expressed in many tissues,[44] and COX-2 is expressed during inflammation and can be induced by extracellular and intracellular stimuli, such as epidermal growth factor (EGF), and this induction is transient.[45-47] In prostate cancer, COX-2 is expressed specifically in prostate inflammatory cells, especially in PIA lesions.[30, 35] Aspirin, also known as acetylsalicylic acid, is unique compared to other NSAIDs. Aspirin is a non-selective COX inhibitor, which irreversibly inhibits both COX-1 and COX-2 iso-enzymes.[48] Aspirin is usually taken for pain, fever, and inflammation treatment. Systemic use of aspirin can prevent strokes, thrombus and heart attacks, and cancers as well, particularly colorectal cancer.[49]

A systematic literature review found that: some studies indicate that aspirin use might be associated with a reduction in prostate cancer risk,[50-63] while others found no protective effect.[64-72] The association between NSAIDs is still unclear due to lack of evidence. Some studies found intake of non-aspirin NSAIDs to be associated with decreased overall prostate cancer risk,[73] however all NSAIDs intake increases prostate cancer risk.[73] Another meta-analysis showed neither adverse or beneficial effects on prostate cancer development or prostate cancer specific mortality, but these results were not consistent.[74] More studies found less consistency when measuring all NSAIDs and could not draw conclusion after meta-analysis.[75, 76] These conflicting findings provide further evidence that aspirin or non-aspirin NSAIDs may have a chemo-preventive effect against prostate cancer and need further epidemiological studies to evaluate their activity.

1.3 PROSTATE CANCER SCREENING AND DIAGNOSIS

Prostate cancer can be found through early detection methods, including: the prostate-specific antigen (PSA) blood test and/or the digital rectal exam (DRE) followed by a prostate biopsy. In Sweden, prostate cancer incidence increased significantly after the PSA testing method was introduced in the 1990s, as was the case in many other Western countries. However, despite this the mortality rate for prostate cancer remained relatively stable. Patients with abnormal PSA or DRE test results receive further biopsies in order to diagnose. Currently, PSA testing can be regarded as an opportunistic screening of prostate cancer.[77] Some prostate cancer will not result in PSA level changes in the blood and only a part of high-level PSA patients will be diagnosed as prostate cancer. At present, the only way to know for certain whether a man has prostate cancer is through biopsy diagnoses.

Prostate cancer can only be cured by radical surgery or radiation when it is still localized. Early diagnosis can provide the best chance at cure. However, present diagnostic and prognostic methods cannot satisfactorily distinguish life-threatening prostate cancer at early stages from the majority of non-harmful indolent tumors. The prostate-specific antigen screening method results in increased incidence of prostate cancer, mostly indolent prostate tumors. This leads to over-diagnosis and over-treatment of patients with non-harmful indolent tumors that can seriously affect their quality of life. An approach called “active surveillance” is employed in monitoring patients with low risk prostate cancer. Doctors and patients have to struggle over the treatment strategy: should they treat directly after diagnosis or utilize active surveillance until disease progresses?

Using detection methods (PSA and DRE), prostate cancer can be diagnosed early on, but it is still debated whether the benefits outweigh risks. The European Randomized Study of Screening for Prostate Cancer (ERSPC) and the Gothenburg trial found that PSA screening decreased mortality from prostate cancer significantly.[78, 79] In contrast, the US Prostate, Lung, Colorectal, and Ovarian cancer screening trial (PLCO) found no mortality reduction benefit from annual PSA screening compared with opportunistic screening for prostate cancer.[80] An observational population-based study (UK ProtecT trial) released findings in October of 2016, stating that prostate cancer specific mortality shows no significant difference across treatments (active surveillance, surgery, or radiotherapy) after a 10-year follow-up, although surgery and radiotherapy reduced disease progression and metastases.[81] More studies are needed to address these conflicting results.

In sum, early detection of prostate cancer is not recommended on the general population scale for the following reasons: 1) its clinical outcomes are not clear, 2) many prostate tumors grow slowly and do not affect life span, and these tests cannot determine which tumors are dangerous, 3) over-treatment has serious adverse effects on patients, and 4) PSA tests result in false-positive and false-negative detection.

1.4 THERAPEUTIC REGIMENS

1.4.1 Prostate cancer disease continuum and corresponding treatments

The clinical manifestation of prostate cancer is very heterogeneous, from harmless slow-growing localized lesions to rapidly progressive metastatic deadly disease. The choice of treatment modalities depends on cancer cell aggressiveness and disease extent as classified using the D'Amico Classification System that based on blood PSA level, prostate biopsy Gleason score and Tumor Node Metastasis (TNM) clinical stage.[82] Patient age and comorbidity play important roles in treatment decision. Standard treatments for prostate cancer patients include active surveillance, radical prostatectomy, radiation therapy, hormone therapy, chemotherapy, and others. (Figure 1).

Autopsy reports revealed that roughly half of all men older than 50 might harbor prostate cancer.[83, 84] The slow growth rate and low possibility of cancer progression make active surveillance or watchful waiting an alternative treatment strategy, which aim to avoid unnecessary treatments, through close monitoring activity. In 2013, active surveillance was selected as treatment for 78% of very low risk and 59% of low risk cancer patients.[85] For intermediate and high risk patients or for localized or locally advanced cancer, radical prostatectomy and curative radiation as well as hormone therapy can be offered (Figure 1). Surgery is a common choice for treating localized prostate cancer. The main type of surgery is radical prostatectomy which has possible side effects of urinary incontinence and erectile dysfunction. Radiation is an alternative treatment option for localized prostate cancer patients. The two most common types of radiation used for prostate cancer are external beam radiation and brachytherapy (internal radiation).

M0 castration-resistant prostate cancer is the cancer stage in which the cancer does not metastasize, but cancer cells are no longer sensitive to hormone treatments. In comparison, M1 hormone-sensitive prostate cancer is the stage where cancer cells have already metastasized but are still sensitive to hormone treatments. There are fewer men diagnosed with M0 castration-resistant prostate cancer than those diagnosed with M1 hormone-sensitive prostate cancer. Treatment regimens are different for the two groups of patients: enzalutamide is the standard treatment for M0 castration-resistant patients, while for M1 hormone-sensitive prostate cancer patients, docetaxel or “abiraterone and prednisone” are the current treatment options [86-88] (Figure 1).

Once metastasis develops, the disease is regarded as incurable. However, several lines of treatments can offer improved palliation and prolonged survival. These include conventional androgen deprivation treatment (ADT) (*e.g.* Luteinizing Hormone-Releasing Hormone (LHRH)-analog or antagonist), next-generation hormone treatment (abiraterone acetate and enzalutamide),[89-91] taxane chemotherapy (docetaxel and cabazitaxel),[92, 93] and radiopharmaceutical agents (*e.g.* Radium 223)[94] (Figure 1).

1.4.2 Hormone treatment and castration/hormone resistance

Hormone therapy (*e.g.* ADT) blocks the production or use of androgen, either by using orchiectomy or by drugs. Removal of the testicles (orchiectomy) has the possibility of decreasing the level of testosterone by 90-95% and it is permanent and irreversible. Luteinizing Hormone-Releasing Hormone agonists/antagonists can significantly reduce the secretion of the luteinizing hormone, which as a consequence suppresses the production of testosterone in the testes. The testosterone drop induced by LHRH agonists/antagonists (also called medical castration or chemical castration) has similar effects as surgical castration (orchiectomy). Unlike orchiectomy, the drug effect on androgen production is reversible. Antiandrogens can inhibit prostate cancer cell growth by competing with androgen for binding to androgen receptors. The production of androgens by adrenal glands and prostate cancer cells can be blocked through Cytochrome P450 17 (CYP17) inhibitors. Cytochrome P450 17 is an enzyme expressed in testicular, adrenal and prostate tumor tissues and can regulate testosterone production.

Androgen deprivation treatment is the standard treatment for prostate cancer patients. Most of these patients respond to ADT initially. However, after 2-3 years, all patients develop castration resistance to ADT (castration-resistant prostate cancers (CRPC)).[95-97] When metastasis occurs (mCRPC), prostate cancer patients have a high risk of dying from the cancer. The median survival time for patients with mCRPCs is up to one and half years from the start of metastasis.[98]

The most common mechanisms causing CRPC are AR-related, including: 1) *AR* amplification, 2) mutated *AR* induced hypersensitivity to ligand binding, 3) co-factors (activators and suppressors) that change AR pathways, 4) ligand independent AR activation, and 5) AR production inside the tumors.[99] However, the androgen signal pathway may not be the only driver in mCRPCs and need further studies to identify, *e.g.* through employing next generation sequencing to find more potential pathways and targets.[98, 100-103]

Next-generation sequencing has been applied to prostate cancer to find genetic alternations in mCRPC, and large-scale data analysis has provided more information for identifying related genetic alternations in cancer progression. Sequencing on paired tumor samples before and after ADT initiation revealed that the *WNT* signaling pathway is a potential target for mCRPC.[104] Mutations, gene expression alternations and fusions were discovered as potential factors in the progression of mCRPC, *e.g. AR, NCOR1* (nuclear receptor corepressor 1), *KDM3A* (lysine demethylase 3A), and new gene fusion *SND1-BRAF* (staphylococcal nuclease domain-containing protein 1 - B-Raf serine/threonine-protein) *etc.*[105] [106] Sequencing of formalin-fixed paraffin-embedded (FFPE) samples may also be a feasible strategy to discover somatic alternations in advanced prostate cancer.[106-108] Circulating free DNA (cfDNA) and circulating tumor cells (CTCs) can also provide a possibility of identifying cancer-related genetic alterations, which would likely benefit patients by assisting in the development of precise personalized treatment.[109] In the near future, tissue-based next-generation sequencing profiling coupled with non-invasive (cfDNA- or CTC-based)

profiling would be a powerful alternative for patients with advanced prostate cancer and would assist in identifying the most promising biomarkers or treatment targets.

1.4.3 Chemotherapy and docetaxel resistance

Common chemotherapies for prostate cancer include docetaxel and cabazitaxel. Docetaxel is the first choice for chemotherapy, and cabazitaxel is additionally utilized if docetaxel does not function or stops working. Both drugs can bind β-tubulin, stabilize microtubule assembly, suppress dynamics of micro-tubules and prevent disassembly, which induces cancer cell death.[110, 111] Cabazitaxel is a second-line chemotherapy treatment with poor affinity for P-glycoprotein compared with docetaxel, which reduces the chances of docetaxel resistance. The median overall survival period can be extended by 3-5 months using docetaxel chemotherapy, while cabazitaxel can result in an additional 2.4 month improvement.[100, 112] Clinical trials have shown that docetaxel can significantly prolong patient survival if it is utilized with ADT in hormone naïve metastatic prostate cancer patients.[93, 113] Clinical experience shows that only approximately half of all patients respond to docetaxel treatment from the start of the treatment, which indicates that around 50% of prostate cancer patients have intrinsic resistance to docetaxel. Those who benefit from docetaxel treatment initially acquire docetaxel resistance eventually.[114, 115] Both intrinsic and acquired resistance result in the failure of docetaxel treatment.

The mechanism behind intrinsic and acquired resistance to docetaxel is not fully understood and there are no clinically reliable biomarkers to predict it to date. Previous research suggests the following mechanisms: 1) high expression/function of drug export pump proteins *e.g.* ABCB1 (ATP binding cassette subfamily B 1), ABCB4, and ABCC1 (ATP binding cassette subfamily C) which could pump out docetaxel from cell plasma and decrease docetaxel concentration,[116] 2) mutations of the corresponding drug targets,[117] 3) inhibition of apoptotic pathways,[118] and 4) altered expression pattern of microtubule-associated proteins and tubulins.[119, 120] Next-generation sequencing of patient-derived pre- and post-docetaxel resistance xenografts showed that SLCO1B3 (organic anion-transporting polypeptide, an influx transporter of docetaxel) was down regulated in resistant xenografts, and intratumoural docetaxel concentrations were significantly decreased after acquiring resistance.[121]

1.4.4 Therapy sequence for CRPC patients

Castration-resistant prostate cancer is a late stage of prostate cancer progression in which the cancer continues to grow even when testosterone is reduced to a very low level *e.g.* castrate level.[122] Metastatic CRPC is one of the major causes of death among patients with prostate cancer. Docetaxel was initially approved as first line chemotherapy for mCRPC patients in 2004, and subsequent drugs approved included abiraterone, enzalutamide, cabazitaxel and Radium-223. The appropriate sequencing of these agents is still not optimized.

Studies have shown that early chemotherapy could improve overall survival for patients with metastatic hormone-sensitive prostate cancer. Several clinical trials were designed to verify

the role of docetaxel in metastatic hormone-sensitive prostate cancer patients, including the CHAARTED and the STAMPEDE studies.[87, 88, 123-125] Data showed that docetaxel could evaluate the effect of hormone treatment and prolong the overall survival of patients with metastatic hormone-sensitive prostate cancer. Radium-223 is unique in reducing skeletal-related events and has been found to prolong survival when utilized after docetaxel treatment.[126] Furthermore, studies showed that Radium-223 has the potential to be utilized in the early stage of prostate cancer *e.g.* before docetaxel.[127]

These data collectively showed that the sequence of treatments for prostate cancer are not yet fixed and optimized. Adjustments could be applied in practical clinical procedures based on new findings of clinical trials.

1.5 THE ROLE OF NON-MUTATED PROTEIN IN PROSTATE CANCER

Genetic robustness is the ability of a biological system to maintain the persistence of a certain trait when facing various external and internal perturbations, such as mutation, recombination or environmental changes. For instance, organisms exhibit a constant phenotype despite of mutation accumulation to a certain extent. Genetic robustness is achieved by a combination of many genes that contribute to a similar function by direct or indirect connection, and *vice versa*, that compensate when perturbations occur in one of these genes (buffering system). Genetic robustness in cancer cells implies that moderate effects and drug resistance occur during treatment when it inhibits only one target (gene/protein). Robustness can be combated through the systematic use of multiple drugs aimed at two or more specific targets, which in combination could result in higher-level effects or even absolute lethality in cancers.[128]

Two or more simultaneous gene defects can cause cell or organism death. This phenomenon is defined as synthetic lethality, and is a consequence of a combination of two or more gene deficiencies where only one of these deficiencies alone does not result in lethality.[129] Mutations, epigenetic alterations or functional inhibitors are effectors that could cause synthetic lethality. Theoretically, when one gene is inhibited by intrinsic gene mutation, if we target and inhibit the other gene of the synthetic gene pair (non-mutated gene), it would result in synthetic lethality. Based on this concept, the paradigm shifts from mutated genes to non-mutated genes as drug targets. The most well-defined example of synthetic lethality is the discovery of PARP inhibitors used in *BRCA* mutated patients with breast cancer, ovarian cancers, and prostate cancer.[130, 131] Experimental data and clinical evidence have shown that *BRCA1* or *BRCA2* and *PARP* are synthetic lethal gene pairs involved in the DNA repair process. Results of clinical trials have shown that *BRCA* deficient patients were sensitive to PARP inhibitors with relatively mild side effects.[132]

Cancer therapies today benefit patients by prolonging their lives by several months to several years. This relatively moderate effect is likely due to 1) only targeting one mutated gene/protein/pathway, while cancer cells are genetically robust and can easily evolve new

alterations to bypass and survive, and 2) to the fact that mutated genes are probably only biomarkers and not driving sources of cancer progression.

A Catalogue of Somatic Mutations (COSMIC, http://cancer.sanger.ac.uk/cosmic) database retrieval shows that, in cancer tissues, prostate cancer has a relatively high non-mutation rate (21.2%) compared with other listed cancer types, *e.g.* breast (10.9%) or liver cancer (12.1%) and that there are higher percentage of mutated genes than non-mutated genes in most of cancer types except in testis cancer (Table 1).

Table 1 Common mutations detected in different human cancer types among the 29,059 genes in total recorded in the COSMIC database.

Cancer Type	Most commonly mutated genes		No. of mutated genes (%)	No. of non-Mutated genes (%)
	1st most (Rate)	*2nd most (Rate)*		
Breast	*PIK3CA* (27%)	*TP53* (24%)	25,888 (89.1%)	3171 (10.9%)
Liver	*TP53* (27%)	*CTNNB1* (22%)	25,541 (87.9%)	3518 (12.1%)
Lung	*TP53* (32%)	*EGFR* (29%)	24,259 (83.5%)	4800 (16.5%)
Kidney	*VHL* (36%)	*PBRM1* (22%)	21,699 (74.7%)	7360 (25.3%)
Ovary	*TP53* (45%)	*FOXL2* (20%)	17,615 (60.6%)	11,444 (39.4%)
Stomach	*TP53* (32%)	*LRP1B* (16%)	24,895 (85.7%)	4164 (14.3%)
Testis	*KIT* (8%)	*KRAS* (4%)	408 (1.4%)	28,651 (98.6%)
Prostate	*TP53* (16%)	*AR* (9%)	22,909 (78.8%)	6150 (21.2%)

It can be hypothesized that some non-mutated proteins may play important roles in maintaining cancer cellular stability. Once mutated, severe cellular instability would be induced to the degree that cancer cells could not survive. These cells, which harbor mutated genes or proteins, could be erased through natural selection during cancer growth. Functional inhibition of these proteins using different targeting methods could be lethal to cancer cells but less harmful to normal cells which generally carry fewer mutations in the genome.

Golgi pH regulator A (GPR89A) is a membrane protein that is localized in the Golgi apparatus and regulates its acidic pH.[133] Research shows that *GPR89A* might be one of the synthetic lethal gene partners of proto-oncogene *MYC*, and data shows that simultaneous knockdown of both genes (*MYC* and *GPR89A*) could induce lethal effects in cancer cells.[134]

1.6 RNA SEQUENCING (RNASEQ)

Next-generation sequencing has revolutionized the field of cancer biology, including prostate cancer. Typical sequencing includes whole-genome sequencing, whole-transcriptome sequencing, whole-exome sequencing *etc*. Single cell sequencing, cfDNA sequencing, and CTC sequencing have also been used to address heterogeneity of tumor tissues. Significant findings have been discovered through next-generation sequencing due to its accuracy and lower cost. However, there are still limitations, *e.g.* quality of sample, sequencing coverage/data quality, and analysis methodology, which all affect the sequencing results.

RNA sequencing is a method that studies the transcriptome, which can be used to discover expression alterations, mutations, fusions *etc*. Illumina is the most widely used technology for RNAseq, as it is sensitive and accurate. Usually, a long gene list with information of interest would be provided after analysis of raw data (reads in fastq file). Pathway/network analysis is employed to find enrichment of a specific important gene group. MetaCore, STRING, PANTHER, DAVID and GeneGo are popular web-based database recourses. Machine learning (both supervised and unsupervised methods) is used to separate sample groups (*e.g.* caner *vs.* normal, localized *vs.* metastasis) to identify genes that contribute most to the group separation. These genes are considered potential biomarkers or targets. A variety of these machine learning techniques, including Artificial Neural Networks, Bayesian Networks, Support Vector Machines, Random Forest and Decision Trees have been widely employed.

2 AIMS

The overall aim of this thesis is to identify potential drug targets and evaluate their role in prostate cancer treatments, as well as to assess the preventive effect of chemo-reagents against prostate cancer.

I. To identify mutations, expression alterations and fusion transcripts as potential drug targets in docetaxel-resistant and castration-resistant prostate cancer.
II. To evaluate the non-mutated protein GPR89A as a potential drug target in prostate cancer.
III. To access the potential preventive effect of maintenance use of aspirin/NSAIDs against prostate cancer based on a nationwide cohort study in Sweden.

3 MATERIALS AND METHODS

3.1 EXPERIMENTAL PROCEDURES

3.1.1 Cell culture

LNCaP, PC3, Du145 and HEK293 cell lines were originally purchased from the American Type Culture Collection (ATCC). The resistant cell line Du145-R was induced and cultured by gradually increasing docetaxel concentrations within the culture medium. Du145-R was cultured in the medium without docetaxel for 30 days to generate a cell line Du145-RB (Table 2).[135]

Table 2 Characteristics of prostate cancer cell lines utilized in this thesis.

Cell lines	Hormone sensitive	Docetaxel sensitive
LNCaP	Yes	Yes
PC3	No	Yes
Du145	No	Yes
Du145-R	No	No
Du145-RB	No	No

Hormone-sensitive: weather the cell line is sensitive to hormone treatment (Yes) or not (No); Docetaxel-sensitive: weather the cell line is sensitive to docetaxel treatment (Yes) or not (No).

3.1.2 RNAseq and data analysis

Total RNA was extracted from cell lines by using TRIzol (Invitrogen, Catalog #15596018) followed by phenol/chloroform extraction procedure. RNase-free DNase set (Qiagen, Catalog #79254) was utilized to remove DNA to avoid DNA contamination. RNA Integrity Number, which was selected as a reference of quality control, was analyzed using the Agilent 2100 Bioanalyzer System. All cell lines were triplicates when sent for sequencing. Total RNA samples were selected by polyA and then clustered on cBot. The sequencing was performed by HiSeq 2000 according to the manufacturer's instructions.

Variant calling: Polymerase chain reaction (PCR) duplicates were removed by Picard (picard.sf.net) and reads were subsequently extracted from the bam file and imported into CLC Genomics workbench (CLC, Aarhus, Denmark). The build 37p5 was utilized as a human reference genome by Large Gap Read Mapping. Probabilistic Variant Detection tool within CLC Genomics workbench was utilized to call variants. Criteria applied during the variant calling procedure were: variant probability higher than 90, minimum coverage and ignore non-specific matches. An alternative method utilized in this thesis was Genome

Analysis Toolkit (GATK) best practices for RNAseq somatic mutation finding. The procedure, which provided step-by-step guiding for performing variant discovery analysis in RNA sequencing data, included base quality score recalibration, INDEL realignment, duplicate removal, and INDEL discovery. RNAseq fastq data was aligned to reference genome hg38 using STAR 2 pass and the GATK was applied to call variants.

Analysis of differentially expressed genes: TopHat was employed to align reads to the reference genome. Cufflinks was utilized to assemble and obtain expression values of genes across genomes. The resulting data was analyzed using Cuffdiff to report the differential expression profile. The CummeRbund R package was utilized for the subsequent analysis and visualization.[136]

Fusion detection: The ChimeraScan was employed to align the paired-end reads to the reference genome. Those read pairs that could not be aligned to the reference genome were trimmed into smaller segments and realigned. [137]

Pathway/network and cluster analysis: Pathway and network analysis were conducted in online services Panther (http://www.pantherdb.org) and Thomson Reuters.[138, 139] The Circos online (http://mkweb.bcgsc.ca/tableviewer) was utilized to visualize fusion transcript distribution among chromosomes. OPLS-DA model was one function of SIMCA software that could be employed to find essential contributors that separated samples into different groups. In our project, two classes (TaxS and TaxR) were pre-set in two groups and VIP scores of each variables/genes were calculated and sorted based on importance.[140]

3.1.3 PCR and quantitative PCR (qPCR)

Total RNA was isolated and extracted by TRIzol (Invitrogen, Catalog # 15596018) following the manufacturer's instructions. Reverse transcription was carried out through Cloned AMV First-Strand Synthesis Kit (Life Technologies, Catalog # 12328). Polymerase chain reaction primers for mutation validation were designed to cover mutation points. Platinum Taq DNA polymerase (Life Technologies, Catalog # 10966018) was utilized to amplify DNA segments. Sanger sequencing was conducted by Eurofins Genomics to confirm mutations found by RNAseq. For validation of up- or down-regulated genes, 20-32 amplifying cycles were selected according to gene expression level. We employed qPCR to validate fusion transcripts. Primers for fusion validation were ordered from Applied Biosystems (Custom plus TaqMan RNA Assays). Roche LightCycler480 SYBR Green I Master (Cat. No. 04887352001) was utilized to conduct qPCR.

3.1.4 Plasmid construction and Western blot

DNA segments of interest were amplified by PCR and digested by restriction enzymes: Sgf I and Mlu I (NEB, Catalog # R0630S and Catalog # R0198S). These digested DNA segments were inserted into the multiple cloning sites of pCMV-AC-GFP (ORIGENE, Catalog # PS100010) by T4 ligase (Promega, Catalog # M180A). Constructed plasmids were

transfected into HEK293 cells and these cells were collected 48 hours after transfection. Anti-TurboGFP antibody (Evrogen, Catalog # AB513) were utilized in Western blot.

3.1.5 Gene knockdown

SiRNAs were synthesized from GE-healthcare (using a customized Cherry pick screening 96 well plate). The plate was stored in the fridge upon arrival and underwent no more than three cycles of thawing-and-freezing. Transfection reagent HiperFect was purchased from Qiagen (Catalog# 301705) and OptiMEM was purchased from Gibco (Catalog 31985). Both were used according to the manufacturer's protocol.

3.1.6 Immunocytochemistry and immunohistochemistry

PC3 cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton™ X-100 for 30 minutes, blocked with 1% BSA for 1 hour and incubated with anti-GPR89A primary antibody (Pierce, Cat. #PA5-33786, 1:400) for 1.5 hours at room temperature. Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody Alexa Fluor® 488 conjugate (Thermo Fisher, Product# A-11008) was diluted (1:400) in phosphate buffered saline containing 0.2% Bovine Serum Albumin (BSA) and incubated for 1 hour at room temperature for detection of the expression level of GPR89A. Formalin-fixed paraffin-embedded tissue was sectioned and deparaffinized using Xylen. Antigen retrieval was conducted in a citrate buffer (pH6, water bath) and the tissue was incubated with 0.5% H_2O_2 in water for 30 minutes. Anti-GPR89A (Pierce, Cat. #PA5-33786) was diluted (1:400) and incubated at 4 °C overnight. Avidin Biotin Complex (30 minutes) was employed to amplify the target antigen signal.

3.1.7 Combination study between docetaxel and *GPR89A* siRNA

Cell seeding and reverse transfection were carried out simultaneously. Docetaxel was utilized to treat cells 16 hours after transfection/cell seeding. Cell viability was measured 48 hours after docetaxel treatment. PrestoBlue (A13261) cell viability reagent was purchased from ThermoFisher. Synergistic effect was determined by comparing 1) the combination effect when docetaxel (D) and *GPR89A* siRNA (G) were used simultaneously to treat cells in the same well ("IR(D+G)") with 2) the estimated additive effect ("IR(D)+IR(G)-IR(D)*IR(G)") where IR(D) indicates the Inhibition Rate (IR) of docetaxel and IR(G) indicates the IR of *GPR89A* siRNA. If the combination effect was higher than the estimated additive effect we concluded that the combinatory treatment of these two reagents could cause a synergistic effect. If the combination effect was equal to or less than the estimated additive effect, we stated that the combinatory treatment of these two reagents could result in an addictive effect or an antagonism effect, respectively.

3.2 REGISTER DATA MANAGEMENT AND STATISTICAL ANALYSIS

3.2.1 Data sources

Sweden is famous for its nationwide, high-quality data registers. Each resident in Sweden has a specific identity number (person number), which makes it possible to link these national registers to each other. In Study IV, we utilized the Swedish Prescribed Drug Register, the Swedish Cancer Register and the Swedish Causes of Death Register.

The Swedish Prescribed Drug Register was established in July 2005 and includes all drugs prescribed and dispensed in Sweden. Over-the-counter drugs and drugs prescribed and dispensed within a hospital are not included. Information from all prescriptions dispensed in ambulatory care is transferred monthly to the National Board of Health and Welfare, which is responsible for the register.[141] The register is complete for the entire Swedish population (patient identification data are missing for <0.3% of all items).[141]

The Swedish Cancer Register was established in 1958 and has the registration of the type and date of diagnosis of all cancers in Sweden since 1961.[142] The completeness of prostate cancer is more than 95% when compared to records of death certificates.[143] This register was used to identify cancer cases among the exposed cohort and the general population. The Registry was also used to exclude individuals with a history of cancer before 2005.

The Swedish Causes of Death Register was established in 1952 and was used to collect date of death among the exposed (maintenance use of NSAIDs) and unexposed individuals. Date of immigration/emigration is not available.

3.2.2 Study design

Study IV is a nationwide Swedish population-based cohort study used to compare the exposed cohort with the Swedish general population of the same age and calendar year.

Study period: The study included individuals who were enrolled between July 1, 2005 (the beginning of the Swedish Prescribed Drug Registry) and December 31, 2012. The study followed individuals until the occurrence of any cancer, death or December 31, 2012, whichever occurred first.

Exposure: Maintenance use of NSAIDs was subdivided as follows. Individuals with ≥180 days of both categories was excluded.

- Aspirin (B01AC06; N02BA), ≥180 days
- Non-aspirin NSAIDs (M01A), ≥180 days

Maintenance use was defined as a cumulative dose of at least 6 months duration during the study period. The total cumulative administered dosage was estimated by the Defined Daily Dose (DDD), which takes the potency of the drug into account as well as the prescribed quantity. The World Health Organisation defines DDD as the assumed average maintenance

dose per day for a drug used for its main indication in adults. Aspirin and NSAIDs are available over-the-counter in Sweden, but only in small packages and at a higher price.

DDD per package= (items issued * amount of drug per item) / DDD

The unexposed reference was general population in Sweden of the same age and calendar period. The fact that many of the individuals in this population use NSAIDs occasionally might to some extent dilute the identified effects, but nonetheless does not explain them.

Outcome: The outcome was a first cancer episode according to the International Classification of Diseases 10th edition.

Potential confounders: Statins have been shown to have potentially protective effects against prostate cancer. The concomitant use of statins (longer than an accumulated 180 days) during the study period was adjusted as a confounder. Age and calendar year were taken into account as well.

Inclusion and exclusion criteria: Only adult men (≥ 18 years) without a previous history of any cancer were included, and only if they had at least 180 days of exposure to aspirin or other NSAIDs (as defined above).

3.2.3 Data collection

The following data was collected from The Swedish Prescribed Drug Register:

- Age at index date (*e.g.* first dispensed prescription of aspirin or NSAIDs during study period) and date of birth.
- Relevant Anatomical Therapeutic Chemical codes.
- Dates of prescribing and dispensing. Date of dispense is considered most accurate since it is closer to actual use.
- To calculate cumulative exposure, the number of prescriptions, dosage, number of doses, DDD were extracted from the register database.

The Cancer Registry was used to collect all prostate cancers cases of interest, as well as exclude individuals with a history of cancer before 2005. Specific detailed variables collected during data management included:

- Date of cancer diagnosis
- Location of tumor and histological type
- Age at diagnosis

3.2.4 Statistical analysis

The risk of developing prostate cancer was compared between the exposed cohort and Swedish general population of the same age by means of standardised incidence ratios (SIRs) and 95% confidence intervals (CIs). The SIRs were calculated by dividing the observed incidence rate of prostate cancer by the expected incidence rate based on the total Swedish

male population of the same age and calendar year.[144] Time of follow-up was from the dispensed date of the first prescription of aspirin/NSAIDs within the study period, until death, cancer or the end of study period, whichever occurred first.

A separate analysis was performed to assess the impact of duration of treatment based on the cumulative dosage (based on sum of DDD) categorized as duration (less than 1 year, 1–3 years, 3–5 years, and longer than 5 years for aspirin, and less than 1 year, 1–3 years, and longer than 3 years for NSAIDs).

4 RESULTS AND DISCUSSION

4.1 DOCETAXEL RESISTANCE IN PROSTATE CANCER (STUDY I)

Triplicates of the docetaxel-resistant (TaxR) cell lines Du145-R and Du145-RB, as well as docetaxel-sensitive (TaxS) cell line Du145, were sent for whole transcriptome RNAseq. Compared to the TaxS cell line, the TaxR cell lines stably acquired 42 mutations. Polymerase chain reaction was employed to validate the accordance between bioinformatics data analysis and wet-lab experiments. Data showed that all the four randomly selected mutations could be validated by PCR, which implied high accuracy of transcriptome sequencing and bioinformatics data analysis. Among these stably acquired mutations in TaxR cell lines, *SMAD4* (Mothers Against Decapentaplegic Homolog 4) and *ABCA2* (ATP-Binding Cassette Sub-family A Member 2), which were previously reported as associated with drug resistance in cancer, were also identified in the TaxR cell lines.[145-148]

Analysis of RNAseq data reported 48, 75 and 66 fusion candidates in the Du145, Du145-R and Du145-RB cell lines respectively. Sixteen fusion candidates whose ChimeraScan score was above 5 were selected to be validated, and among them, 13 (81.25%) could be verified by PCR and Sanger sequencing. Of the 13 chosen validated fusion candidates, 10 were commonly expressed in all the three cell lines. One fusion (*MYH9-EIF3D*) was exclusively identified in the TaxR cell line and two other fusions (*TAF15-AP2B1, VCL-ADK*) were only detected in the TaxS cell line, which implies its potential association with primary or acquired resistance to docetaxel.

Four fusion transcripts (*MYH9-EIF3D, LDLR-RPL31P11, TAF15-AP2B1, VCL-ADK*) were selected to be validated by qPCR and Western blot. Data showed that all the four fusions could be transcribed and translated in the plasmid transfected HEK293 cell line. The fusion transcripts were validated by qPCR in the original cell lines (Du145, Du145-R and Du145-RB), however, these fusion proteins could not be detected in the cell lines using Western blot, which was possibly due to low expression. Interestingly, we found two bands in one PCR lane when *VCL-ADK* was amplified, which indicated different transcript patterns of these two proteins. Sanger sequencing showed that both of the fusions were *VCL-ADK* fusion transcripts, although with different fusion points within *ADK*.

In total, we identified 329 up-regulated and 286 down-regulated genes whose expressions were altered in Du145-R as well as in Du145-RB as compared to Du145. These genes demonstrated stably altered expressions when the cells acquired resistance to docetaxel. The 40 most up- or down-regulated genes in the TaxR cell lines were selected and further validated by PCR. Of these genes, 37 out of 40 (92.5 %) were found in accordance with the bioinformatics data analysis of RNA sequencing. Among the up-regulated genes, *ABCB1*, which was reported as a transporter of small molecular drugs through membranes and associated with drug resistance,[147] was also identified in this project. Moreover, pathway and network analysis reported three knots: *NF-κb* (Nuclear Factor Kappa B), *EGR1* (Early Growth Response 1) and *ETS*, through which most of the genes with altered expression were

linked to the whole network. The importance of *ABCB1* was further confirmed through its connection to these three knots in the network analysis.

4.2 CASTRATION RESISTANCE IN PROSTATE CANCER (STUDY II)

To identify acquired mutations, altered expressions, and the existence of fusion transcripts during the development of castration resistance, we performed whole transcriptome RNAseq in triplicates of two castration-resistant cell lines, PC3 and Du145, and one hormone-sensitive cell line LNCaP. After analysis, we identified 4397 mutations (in 2579 genes) that were acquired when cells became resistant to hormone treatment. Pathway analysis revealed that the most enriched pathway was the immune response B cell antigen receptor (*BCR*) pathway, which indicates a potential immune system related mechanism behind acquired resistance to hormone treatment.

Compared to the hormone-sensitive cell line, we identified 157 down-regulated and 549 up-regulated genes whose expressions were consistently regulated in both of the castration-resistant cell lines. Thirty of the dysregulated genes were selected for validation by PCR. Results showed that 28 out of 30 could be verified, indicating a high quality of the RNA sequencing analysis. Furthermore, all up- or down-regulated genes (in total 706) were analyzed using network enrichment, which reported three dominant connection knots: *GCR* (*NR3C1,* Glucocorticoid Receptor), PKA-cat kinase (*PRKACB*, cAMP-dependent Protein Kinase Catalytic Subunit Beta) and protein kinase C family (*PRKD1,* Serine/Threonine-Protein Kinase D1). The glucocorticoid receptor has previously been reported as associated with acquired resistance to ADT in prostate cancer.[149-151] Moreover, PRKACB and PRKD1 are involved in various cellular functions *e.g.* proliferation, differentiation and apoptosis, which implies their potential importance in cancer progression.

We also identified 117, 48 and 60 fusions in the LNCaP, Du145 and PC3 cell lines, respectively. Among them, one fusion (*AF086285-ATP6V1E2*) was commonly detected in all three cell lines and six were identified exclusively in the castration-resistant cell lines. Out of 25 selected chimeric transcripts (unique alignment positions parameter > 5), 11 top-ranked fusions were selected and data showed that eight of these (72.7%) could be validated by PCR. Among them, four chimeric transcripts (*MIPOL1-DGKB*, *GPS2-MPP2*, *RERE-PIK3CD* and *TFDP1-GRK1*) could only be detected in the hormone-sensitive cell line LNCaP, while the other three (*SMAGPTFCP2*, *KDM5B-CR936711*, *SAMD8-ADK*) were only detected in the castration-resistant cell line. These findings collectively indicate that the fusions exclusively expressed in the castration-sensitive or resistant cell lines respectively might be associated with the primary or acquired resistance to hormone treatment. However, further experimental validations are needed.

Multiple molecular mechanisms are involved in the development of castration resistance, and the most well-defined mechanism is dysregulations of AR. This study demonstrated that AR was not expressed in the two hormone-resistant cell lines, PC3 and Du145, which was in

agreement with findings from previous publications.[152-154] This might therefore indicate that the alterations acquired in the castration-resistant cell lines in this study were possibly AR-independent.

4.3 EVALUATION OF THE NON-MUTATED PROTEIN AS A POTENTIAL DRUG TARGET IN PROSTATE CANCER (STUDY III)

To assess the percentage of mutated genes in prostate cancer, a retrieval was performed in the COSMIC database and 22,909 genes were identified as mutated (78.8%) out of all genes recorded (29,059). The remaining 6150 (21.2%) genes were not reported as mutated. Comparing this with our RNAseq data of prostate cancer cell lines in Study I and Study II, we selected 17 genes which had higher mRNA expression. Among these selected genes, knockdown of *GPR89A* was associated with decreased cell viability as well as increased apoptosis in both PC3 and Du145 prostate cancer cell lines and therefore was selected as the gene of interest.

Immunocytochemistry showed that GPR89A was expressed in cytoplasm in the prostate cancer cell line PC3. Immunohistochemistry in prostate cancer patient tissues indicated that the cancer cells within tumor areas showed a higher GPR89A expression compared to adjacent benign areas.

Docetaxel is a standard treatment for metastatic prostate cancer. To evaluate the combinational potential between docetaxel and *GPR89A* siRNA, we measured the effect of combined treatment of docetaxel and *GPR89A* siRNA on PC3 cell line compared to their estimated additive effect. Based on concentration titration, single reagent effect of docetaxel at 0.3 nM and *GPR89A* siRNA at 5-80 nM were selected in the combination study. The corresponding estimated additive effect for each concentration was calculated. Combined treatment of docetaxel and *GPR89A* siRNA showed higher effect than the estimated additive effect, which indicated that docetaxel has a synergistic combination effect with all different concentrations of *GPR89A* siRNA ranging from 5 nM to 80 nM.

The importance of the Golgi apparatus has become more and more evident as a potential anti-cancer target.[155, 156] Previous studies have demonstrated the potential of Golgi-associated molecules as drug targets in androgen-sensitive and resistant prostate cancers.[155] Notably, the protein of interest GPR89A is localized in the Golgi apparatus and functions in pH regulation of the internal Golgi environment. This might be the mechanism of action of GPR89A as a potential drug target in prostate cancer. Moreover, docetaxel is an anti-mitotic chemotherapy functioning through microtubule stabilization, which enhances microtubule polymerization and affects cancer cell survival. The combination treatment of docetaxel and knockdown of *GPR89A* targets both of the essential intracellular systems and causes dysfunction of Golgi as well as microtubules, which might eventually result in synergistic effects.

4.4 ASPIRIN/NSAIDS AND PROSTATE CANCER PREVENTION (STUDY IV)

The prevention effect of aspirin and non-aspirin NSAIDs was estimated by including 419,931 aspirin users in the cohort which contributed 2,053,932 person-years, as well as 223,437 non-aspirin NSAIDs users which contributed 1,305,848 person-years. Aspirin users were older and had a higher percentage of prostate cancer cases than non-aspirin NSAIDs users. Among aspirin users, 62.9% of men had concomitant maintenance use of statins, while only 14.7% non-aspirin NSAIDs users were found to have this concomitance.

The overall SIR of aspirin users is 0.87 with 95% CI 0.85–0.88, which suggests that maintenance aspirin intake could protect men from prostate cancer. All age groups above 50 showed a reduced risk of prostate cancer. The NSAIDs also showed a preventive effect against prostate cancer (SIR=0.87, 95% CI 0.85–0.90). Yet, the association was only significant in the age groups of 50–59 and 60–69 years.

Long-term intake of aspirin (longer than five years) showed a significant protective effect (SIR=0.31 95% CI 0.30–0.32), while shorter duration groups indicated a lower protective effect. In the non-aspirin NSAIDs group, the protective effect appeared from intake of one year and longer, and the strongest preventive effect was found in the subgroup of those taking NSAIDs for more than three years (SIR = 0.58, 95% CI 0.53–0.63).

When the cohort was separated according to the status of concomitant maintenance use of statins, no significant effect remained in the aspirin group without concomitant statins intake (SIR=0.99, 95% CI 0.96–1.02), except for after long-term exposure (SIR=0.31, 95% CI 0.29-0.34). Maintenance use of non-aspirin NSAIDs did show a protective effect, although this was less pronounced when maintenance statins users were excluded (SIR = 0.92, 95% CI 0.88–0.95). Poisson regression adjusted for age showed that statins intake reduced the risk of prostate cancer by 22% (IRR=0.78, 95% CI 0.75–0.81) in the aspirin group and by 20% (IRR = 0.80, 95% CI 0.73, 0.87) in the non-aspirin NSAIDs group, compared to non-statins users.

These findings collectively suggest an overall protective effect of aspirin and non-aspirin NSAIDs against prostate cancer, especially after long-term intake. The effect of aspirin was higher than non-aspirin NSAIDs, however, this influence could be explained to some extent by concomitant statins intake among 63% of aspirin users.

This is a nationwide cohort study based on the Swedish population. The register databases of the entire Swedish population provided accurate and valid data sources, especially the Prescribed Drug Register, which decreased recall bias and misclassification of exposures. A minor limitation of the drug register is that it does not include drugs purchased over-the-counter. However, prescribed drugs in Sweden are subsidized and cheaper, so we therefore assumed that the majority of drugs were purchased through a prescription. We assessed drug usage by average DDD per package, which might induce an over- or under-estimation of the accurate daily use.

In this project, statins were found to be associated with a reduced risk of prostate cancer, and the protective effect of aspirin was diluted by concomitant maintenance use of statins. Unfortunately, we could not measure the interaction between aspirin/non-aspirin NSAIDs and statins due to the study design. Some confounders *e.g.* obesity, diet, and socio-economic and ethno-geographic risk factors were not included in this analysis. However, Sweden is a fairly small and developed country, and there is a relatively low level of differences in socio-economic status and ethnicity for the majority of inhabitants.

5 PERSPECTIVES

Current clinical challenges in prostate cancer includes: 1) inevitable drug resistance to a variety of treatments, 2) lack of accurate diagnostic and prognostic biomarkers, as well as 3) no common chemoprevention strategies. This thesis is multi-dimensional and addressed different aspects of prostate cancer research areas, from castration resistance to docetaxel resistance to cancer prevention, by identifying transcript alternations associated with drug resistance and estimating the prevention effect of aspirin and non-aspirin NSAIDs against prostate cancer.

Genomic alteration is one of the hallmarks of human malignancies, including mutations, altered gene expressions and fusion transcripts. We identified a variety of transcript alterations that were possibly associated with either hormonal resistance or docetaxel resistance. However, these genomic/transcriptomic alterations occur during cancer progression and their roles are still controversial: Are they driving sources or only concomitant phenomenon during cancer progression? Additionally, the percentage of mutated genes in cancers showed that only about 20% of all genes have never been reported as mutated in prostate cancer. This might imply that the majority of these mutated genes are passengers not drivers of cancer progression. Our findings in Study III suggest a new research area, which is to treat cancer by targeting non-mutated genes that might play an essential role in maintaining the stability of cancer cells.

There is a saying that “prevention is better than a cure”. However, to date this can only be applied in a minority of cancers *e.g.* cervical cancer prevention by HPV vaccines and breast cancer prevention by surgically removing breast tissues. Recently, aspirin was recommended in the US to prevent colorectal cancer. For other cancer types, including prostate cancer, there are no commonly known preventive strategies. Study IV elucidates the potential of aspirin and non-aspirin NSAIDs as preventive reagents in prostate cancer. However, further studies are still needed to accumulate evidence either in support of this approach or to identify new protecting reagents.

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